Molecular Investigations into Wiskott-Aldrich Syndrome

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Abstract

Wiskott-Aldrich Syndrome (WAS) is a rare X-linked recessive primary immunodeficiency characterised by eczema, thrombocytopaenia and immunodeficiency. WAS encodes for a haematopoietic restricted protein, WASp, involved in transduction of signals from the cell membrane to the actin cytoskeleton. Mutations lead to impaired actin dynamics in response to stimuli and are seen as defects of receptor capping, chemotaxis, phagocytosis and proliferation. This leads to susceptibility to pyogenic, viral and opportunistic infections and increased incidence of lymphoproliferative disease and malignancy. Due to the high morbidity and mortality associated with mismatched transplantation, WAS is considered a good target for gene therapy. We were able to reconstitute a murine model of WAS using a gamma retroviral vector, with reconstitution of specialised actin structures, podosomes, as a functional readout. Reservations concerning the safety of such vectors, following adverse events in a clinical trial, led to the development of third generation self-inactivating lentiviral vectors. Under the promotion of CMV, SFFV LTR or short sequences of the proximal endogenous Wiskott-promoter, WASp was able to restore cytoskeletal abnormalities in dendritic cells *in-vitro* from WASp-deficient mice. In addition we have demonstrated stable expression of WASp in T cell, B cell and myeloid lineages following transduction and engraftment of lineage negative murine bone marrow (up to 9 months) using both an SFFV LTR and endogenous promoter sequences. We have demonstrated the ability to restore cytoskeletal abnormalities and proliferative responses from the reconstituted mice. Recently a novel mutation in WAS has led to the discovery of a constitutively active WASp, with a novel monocytopaenic and neutropaenic phenotype. Here we characterised the patient phenotype of a novel constitutively active $WASp^{I294T}$ mutant. In addition to monocytopaenia and neutropaenia, there was abnormal cytoskeletal actin, manifesting itself as abnormal podosome distribution and an inability to phagocytose or produce an oxidative burst to physiological stimuli. There was also increased apoptosis in the bone marrow and evidence of genomic instability. Utilising lentiviral vectors, $WASp^{I294T}$ was expressed in a cell line to elucidate the possible mechanisms responsible for the patient phenotype. These studies demonstrate the efficacy and feasibility of a lentiviral vector-mediated gene therapy strategy for WAS using endogenous promoters where a more regulated expression level may be achieved.

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Abbreviations

7-AAD	7-amino-actinomycin D
AAV	Adeno-associated virus
ADA	Adenosine deaminase
Bm	Bone marrow
BMT	Bone marrow transplant
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CMV	Cytomegalovirus
cPPT	Central poly purine tract
CSF-1	Colony stimulating factor-1
Cy-5	Cy-chrome-5
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNFB	Dinitrofluorobenzene
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EF1-α	Elongation factor-1-alpha
ELISA	Enzyme linked immunoabsorbent assay
Env	Envelope
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
fMLP	f-Met-Leu-Phe
Gag	Group specific antigens
GM-CSF	Granulocyte macrophage colony stimulating factor
HIV-1	Human Immunodeficiency Virus -1
HSC	Haematopoietic stem cell
HSV	Herpes Simplex Virus
Ig	Immunoglobulin
IL-3	Interleukin-3
IL-6	interleukin-6
IRES	Internal ribosomal entry site
kD	Kilo Daltons
LTR	Long terminal repeat
MACS	Magnetic Active cell sorting
M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
MMLV	Molony Murine Leukaemia Virus
MMP	Matrix Metallo Proteinases
MOI	Multiplicity of infection
NK	Natural Killer
N-WASp	Neural WASp
PAGE	Polyacrylamide gel electrophoresis

PBL	Peripheral blood leukocytes
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phyto haemagglutinin
PI	Propiduim odide
PMA	Phorbo-12-myriste-13-acetate
Poly A	Poly adenylation
Pgk	Phosphoglycerate kinase
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Rous Sarcoma virus
RT-PCR	Real-Time PCR
Sca 1	Stem cell antigen 1
Scar	Suppressor of cAR
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SFFV	Spleen focus forming virus
Spl	Spleen
S/NOD	SCID/ Non-Obese Diabetic
Thy	Thymus
TPO	Thrombopoeitin
VSV.G	Vasicular stomatitis virus glycoprotein
v/v	Volume to volume
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WAVE	WASp family Verprolin homologous protein
WIP	Wiskott Interacting Protein
Wp	Wiskott promoter
WPRE	Woodchuck hepatits virus post-transcriptional regulatory element
XLT	X-Linked Thrombocytopaenia
X-SCID	X-linked severe combined immunodeficiency

CHAPTER 1

INTRODUCTION

1. Introduction

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive primary immunodeficiency. It was first described by Wiskott, in 1936, when patients presented with symptoms of thrombocytopaenia, bloody diarrhoea, eczema and recurrent inner ear infections. Subsequently in 1954, Aldrich described affected males and concluded the disease was X-linked recessive. Since these early diagnoses, the triad of eczema, immunodeficiency and thrombocytopaenia with small platelets now describes the syndrome. Due to variability of symptoms however, this triad is actually only seen in 30% of cases, with the thrombocytopaenia the least variable symptom. In addition there are recurrent bacterial and viral infections and increased risk of malignancies. Overall there is a high level of variability in the symptoms and milder forms of WAS are often classified as X-linked thrombocytopenia (XLT) although both have been shown to be due to mutations in the WAS gene (Villa et al., 1995; Zhu et al., 1995).

1.1 Description of Disease

Patients have microthrombocytopaenia and as a result have the associated problems of bleeding and haemorrhaging, which accounts for approximately 23% of deaths associated with disease (Sullivan et al., 1994).

Immunological defects associated with mutations in the WAS gene affect both the lymphoid and myeloid compartments of the haematopoietic system. These include an impairment of delayed hypersensitivity and mitogen responses and failure to respond to polysaccharide antigens (Ochs et al., 1980; Lo et al., 1999a). There is low serum IgM and IgG with increased levels of IgA and IgE, and responses to some proteins do not show class switching (Ochs et al., 1980). The total numbers of circulating lymphocytes

in infants is not reduced, but they often develop lymphopaenia by 6-8 years of age, which has been attributed to increased levels of apoptosis (Rawlings et al., 1999).

This immune disorder in WAS leads to susceptibility to pyogenic, viral and opportunistic infections and an increased incidence of lymphoproliferative disease and malignancy, accounting for 44% and 26% of deaths, respectively (Sullivan et al., 1994). In fact due to improved medical care the median survival is now around 15 years of age but with this increased life span an increase in malignancies has been observed.

1.2 Genetics of WAS

WAS is an X-linked recessive disease affecting 1 in 250,000 live births in Europe (Thrasher and Kinnon, 2000b). The gene responsible was cloned in 1994 and found to lie on the short arm of the X chromosome at Xp11.22-p11.23 (Derry et al., 1994). It is comprised of 12 exons over 9kb of genomic DNA. The cDNA encodes a 502 amino acid protein, WASp, with a molecular weight of approximately 65kD, with expression restricted to cells of the haematopoietic lineage (Derry et al., 1994; Stewart et al., 1996). It is mutations in this gene that lead to Wiskott-Aldrich syndrome.

1.2.1 Mutations in WAS

In addition to Wiskott-Aldrich syndrome, it has subsequently been shown that mutations in WAS can also cause X-linked thrombocytopaenia (Villa et al., 1995; Zhu et al., 1995). Investigations into the positions of the mutations and the severity of disease have revealed mixed results. Schindelhauer *et al.* found no correlation between genotype and phenotype (Schindelhauer et al., 1996), as did others (Greer et al., 1996), conflictingly however Zhu *et al.* found that missense mutations in the first three exons

generally resulted in a mild disease phenotype and reduced WASp expression (Zhu et al., 1997), whereas more complex mutations and missense mutations in the remainder of the gene result in lower protein levels (if any) and a more severe phenotype. A recent more comprehensive study of 262 patients from North America and Europe (Jin et al., 2004) found five mutational hotspots and a strong association between genotype and phenotype. It was found that a less severe disease phenotype was seen in patients with mutations which allow some protein expression e.g. missense, whereas patients with more complex mutations which lead to no protein expression revealed a more severe phenotype.

Recently an incidence of congenital X-linked neutropaenia was caused by a mutation in the Cdc42 binding domain of *WAS* (Devriendt et al., 2001) without the thrombocytopaenia associated with classical WAS, further showing the complicated link between genotype and phenotype.

1.2.2 X-inactivation

Heterozygous female carriers of a mutated *WAS* gene have been shown to undergo apparent non random X-inactivation in all haematopoietic lineages (Greer et al., 1989), even in CD34 positive stem cells (Wengler et al., 1995) from which all mature blood cells derive, suggesting WASp expression is critical for survival of mature and primitive haematopoietic cells and possibly their development. Similar phenomena have been seen in other X-linked diseases and are consistent with a selective advantage for cells without the mutation.

1.2.3 Female WAS patients

As with any X-linked disease it is extremely unusual for females to be affected. Nevertheless, there have been some females with clinical symptoms of WAS. In one case there was skewing of the X inactivation by allowing the spontaneously mutated paternal X chromosome to be activated (Parolini et al., 1998), possibly through a XIST mutation. In other cases skewing of X inactivation is thought to be the underlying reason for a disease phenotype (Kondoh et al., 1997; Luzzatto and Martini, 1998). Female XLT phenotypes have been reported (Inoue et al., 2002; Proust et al., 2005) showing the type of mutation is still important in determining the severity of disease.

1.2.4 Somatic Mosaicism

Mosaicism occurs when there is the spontaneous reversion from a disease phenotype to a normal or mild phenotype by *de novo* mutations leading to 2 or more populations of cells. Because of the variation in the disease phenotype compared to genetic mutations discussed earlier, reversions are difficult to diagnose. However there have been three cases (Ariga et al., 1999; Ariga et al., 2001; Wada et al., 2003) reported to date; 1. Where there is a single nucleotide reversion, 2. An insertion event restoring the open reading frame, and 3. A deletion of 6bp reinserted, have been described. Only one case (Wada et al., 2001) had a definite improvement of disease phenotype upon reversion of the mutation even though all had a partial restoration of the T cell compartment showing a selective advantage. The lack of improvement in disease phenotype seen may be due to irreversible damage to the body, as the timing of the reversions could not be measured. Additionally, normal T cells alone may not alleviate symptoms and myeloid cells may be more responsible for the immune dysregulation seen in WAS patients. Somatic mosaicism has been seen in other X-linked diseases such as Adenosine Deaminase Deficiency (ADA) (Hirschhorn et al., 1996) and X-linked

Severe Combined Immunodeficiency (SCID) (Stephan et al., 1996). In the case of X-linked SCID a reversion mutation in the common γ chain in an early T cell progenitor led to a normal phenotype creating a partially restored T cell compartment (Stephan et al., 1996) due to a selective advantage. Somatic mosaicism is encouraging for treatment of WAS by gene therapy because a selective advantage would be predicted in transduced T cells, although B and myeloid cells may need higher levels of transduction as there is no selective advantage (Konno et al., 2004). A selective advantage seen with transduced T cells in X-linked SCID has aided the correction of the disease, even with relatively low levels of transduction.

1.3 Domain Structure of Wiskott-Aldrich syndrome Protein

WASp is a predominately cytosolic protein which is excluded from the nucleus (Rivero-Lezcano et al., 1995) and is part of a family of related proteins. Other members of the family are more ubiquitously expressed, but all are involved in the transduction of signals from the cell surface to the actin cytoskeleton and all can induce actin polymerisation *in vitro* (Zalevsky et al., 2001). WASp can be divided into specific domains that have different functions and homology (**Figure 1.1**). Other members of the family include N-WASp, Scar/WAVE, and Bee1p as well as WASp homologues in different species (**Figure 1.2**) and can also be divided into similar domains. They all possess a VCA (verprolin/cofilin homology, acidic region) domain and a proline rich domain at the C-terminus (**Figure 1.2**). WASp and N-WASp have a GTPase binding domain (GBD) although this is not found in Scar/WAVE (**Figure 1.2**). Another difference with WAVE is in the N-terminus, as there is a WAVE homology domain instead of a WASp homology domain (**Figure 1.2**). The main members of the WASp

family will be discussed in more detail followed by the homologues and their differences.

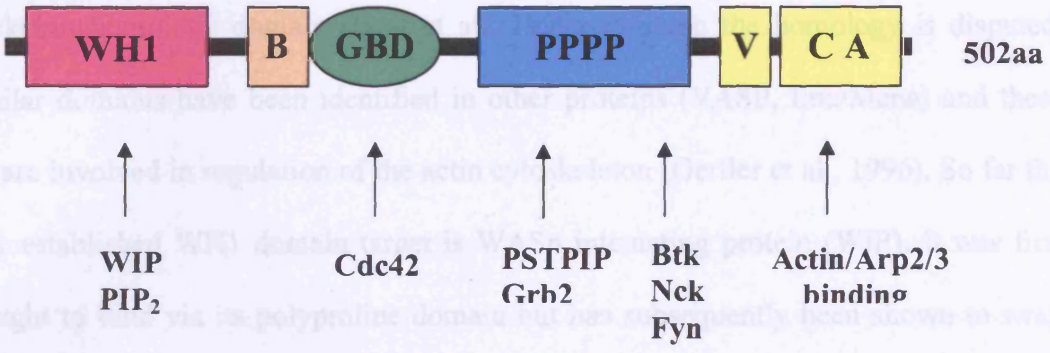


Figure 1.1 Domain structure of WASp
Cartoon of Wiskott-Aldrich syndrome protein showing the various domains determined by function, homologies, and known protein-protein interactions. The N-terminus WASp Homology 1 (WH1) domain binds Wiskott-Interacting protein (WIP) and PIP_2. There is a central GTPase binding domain (GBD) thought to be important in the regulation of WASp and a polyproline domain (PPPP) through which there are protein/protein interactions from cytosolic signalling molecules. The C-terminus verprolin-cofilin acidic domain (VCA), often also named the WH2 domain, binds p21arc of the arp2/3 complex and monomeric actin and has been implicated in the initiation of dendritic branching of filamentous actin.

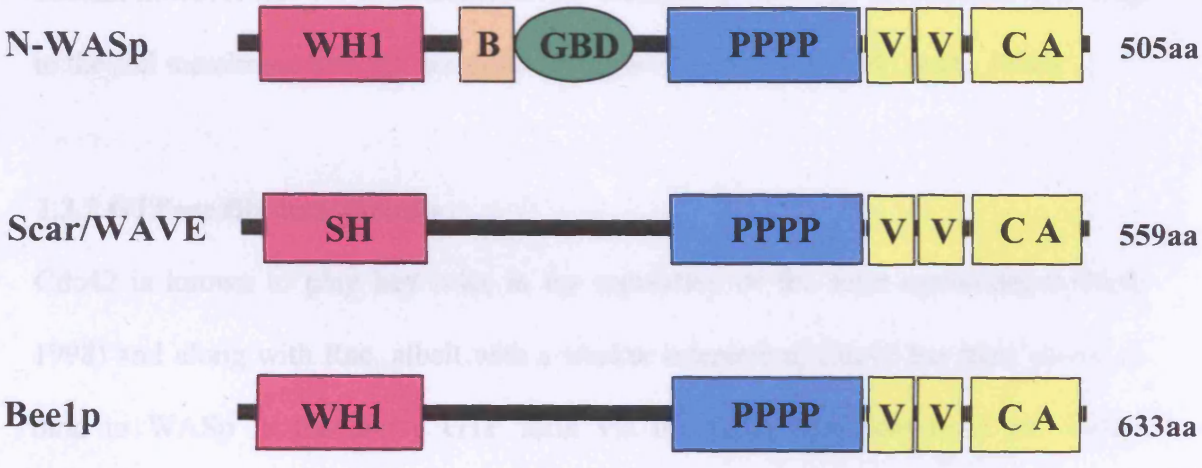


Figure 1.2 WASp family members

Cartoon representations of other WASp family members showing the domain structure defined by function, homologies and known protein/protein interactions. The ubiquitous N-WASp and yeast homologue Bee1p have an N-terminal WASp homology 1 (WH1) domain, whereas the Scar/WAVE proteins (1-3) have a Scar homology domain (SH). N-WASp has a GTPase binding domain (GBD) but is absent in WAVE and Bee1p. All three proteins contain a central polyproline (PPPP) containing domain similar to WASp. Another difference from WASp is the presence of additional verprolin homology domains at the C-terminus.

1.3.1 WH-1 Domain of WASp

This is the least understood of all the domains of WASp and has been referred to as a pleckstrin homology domain (Imai et al., 1999) although the homology is disputed. Similar domains have been identified in other proteins (VASP, Ena/Mena) and these too are involved in regulation of the actin cytoskeleton (Gertler et al., 1996). So far the only established WH1 domain target is WASp interacting protein (WIP). It was first thought to bind via its polyproline domain but has subsequently been shown to wrap around the entire WH1 domain (Volkman et al., 2002). The majority of mutations in *WAS* are in the WH1 domain and may alter the binding of WIP and be important in the regulation and stability of WASp. However, severity of disease is variable even with similar mutations in this domain suggesting transcriptional activating ability through this domain may be differently affected (Silvin et al., 2001). The relevance of the WH1 domain however has yet to be conclusively elucidated, although localisation of WASp to the cell membrane through this domain has been postulated (Miki et al., 1996).

1.3.2 GTPase Binding Domain

Cdc42 is known to play key roles in the regulation of the actin cytoskeleton (Hall, 1998) and along with Rac, albeit with a weaker interaction, Cdc42 has been shown to bind to WASp in the active GTP form via the GBD (Aspenstrom et al., 1996). Subsequently it was revealed extra WASp sequences outside the GBD are required for tight binding (Rudolph et al., 1998). This data along with the abnormal actin polymerisation when a dominant negative form of Cdc42 is co-expressed (Symons et al., 1996) leads to the conclusion that WASp is a downstream effector of Cdc42.

When activated Cdc42 binds to WASp, in conjunction with PIP_2, there is a change in the structure of WASp from an autoinhibited state to an active state (Kim et al., 2000)

releasing the proline rich domain and VCA domain leading to intracellular changes in actin (**Figure 1.3**). Recently a mutation in WASp was identified in the GBD region, and is thought to prevent the autoinhibited confirmation of WASp. This mimics binding of the GTP form of Cdc42, resulting in severe neutropaenia and constitutive actin polymerisation *in vitro* (Devriendt et al., 2001) .

Although a key role for Cdc42 binding to WASp thus subsequently affecting the activity of WASp seems likely, there is evidence of actin clustering and recruitment of WASp to an immunological synapse independent of Cdc42 (Kato et al., 1999; Cannon and Burkhardt, 2004; Cannon et al., 2001), suggesting there are other influences and possibly other mechanisms for WASp activation (Badour et al., 2003b).

1.3.3 Proline Rich Domain

WASp has a proline rich domain that has been shown by many groups to be capable of binding the SH3 domains of other cytosolic proteins. These proline rich domains are found in all members of the WASp family and are thought to provide a scaffold to allow interactions between signalling molecules. Amongst the proteins found to bind *in vivo* are tyrosine kinases and adaptors including Nck (Rivero-Lezcano et al., 1995), PSTPIP1 (Wu et al., 1998), Grb 2 (She et al., 1997), Fyn (Banin et al., 1999) and Btk (Baba et al., 1999). These binding partners have been found to be involved in the regulation of the actin cytoskeleton, either upon growth factor stimulation (Matuoka et al., 1993) or in cytokinesis (Spencer et al., 1997).

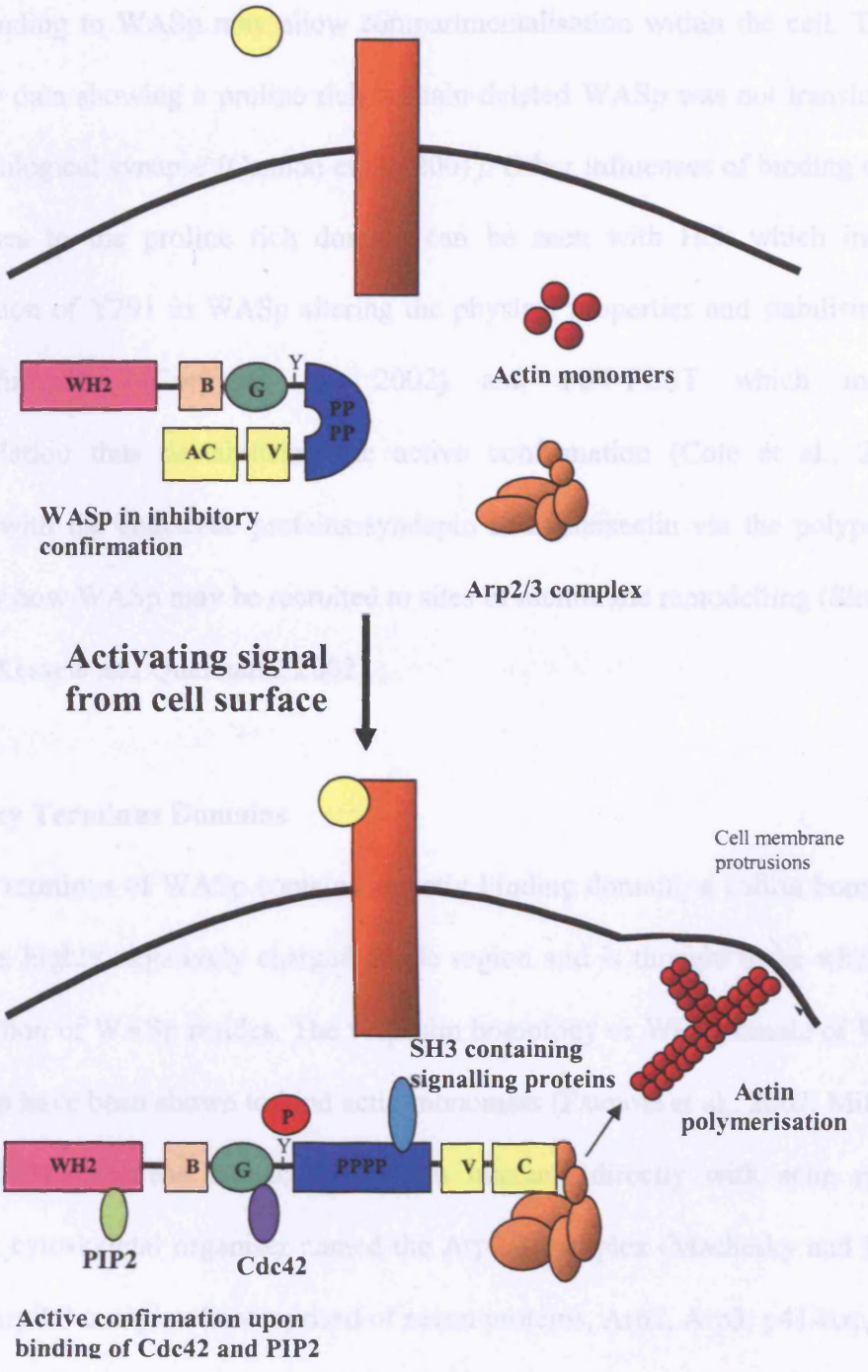


Figure 1.3 Actin of PIP_2 and Cdc42 on activation state of WASp
Cdc42 and PIP2 bind to WASp causing the release of the inhibited WASp conformation This allows the binding of SH3 containing proteins and the phosphorylation of tyrosine 291 stabilising the active conformation and allowing response to specific stimuli. Unmasking of the VCA Arp2/3 binding domain allows polymerisation of actin monomers to actively progress allowing membrane protrusions e.g filopodia and phagocytic cups to form.

Therefore binding to WASp may allow compartmentalisation within the cell. This is supported by data showing a proline rich domain-deleted WASp was not translocated to an immunological synapse (Cannon et al., 2001). Other influences of binding of Src family kinases to the proline rich domain can be seen with Hck which induces phosphorylation of Y291 in WASp altering the physical properties and stabilising the active confirmation (Cory et al., 2002) and PST-PEST which induces dephosphorylation thus destabilising the active confirmation (Cote et al., 2002). Interactions with the endocytic proteins syndapin and intersectin via the polyproline domain show how WASp may be recruited to sites of membrane remodelling (Simpson et al., 1999; Kessels and Qualmann, 2002).

1.3.4 Carboxy Terminus Domains

The carboxy terminus of WASp contains an actin binding domain, a cofilin homology domain and a highly negatively charged acidic region and is thought to be where the effector function of WASp resides. The verprolin homology or WH2 domain of WASp and N-WASp have been shown to bind actin monomers (Paunola et al., 2002; Miki and Takenawa, 1998) whereas the acidic domain interacts directly with actin related proteins of a cytoskeletal organiser named the Arp2/3 complex (Machesky and Insall, 1998). The Arp2/3 complex is comprised of seven proteins, Arp2, Arp3, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc and has been shown to be localised to regions of dynamic actin polymerisation such as filopodia and lamellipodia in cells (Welch et al., 1997).

Overexpression of the C-terminus domain of WASp results in dysregulation of the actin cytoskeleton (Symons et al., 1996) and it is thought that WASp acts as a cellular

actin nucleation promoting factor (Higgs et al., 1999). This nucleation activity provides a molecular explanation for the role of WASp in transduction of signals from the cell surface by holding together cytosolic proteins leading to actin remodelling within a cell.

In addition to binding Arp2/3, the acidic domain of WASp interacts with the basic domain to hide the VCA and proline rich domain (**Figure 1.3**). This results in an inhibitory confirmation, which is released upon PIP_2 and Cdc42 binding (Kim et al., 2000). This releases the C terminus to allow actin remodelling and in fact the mutant of WASp, which results in an active protein conformation, polymerises actin constitutively (Devriendt et al., 2001).

The lack of transduction of signal mediated by WASp may be the cause of some of the defects seen in patients that lack WASp. The amelioration of phenotype in XLT may be due the presence of low levels of cellular WASp allowing some signalling to occur.

1.4 WASp Family Members

1.4.1 N-WASp

As has been discussed earlier, WASp expression is restricted to cells of the haematopoietic lineage, whereas N-WASp (Neural-WASp) (**Figure 1.2**), although first found in the brain, has a more widespread distribution (Miki et al., 1996). There is 50% homology between the two proteins with N-WASp possessing an extra verprolin homology domain. N-WASp is thought to be involved in the formation of filopodia (Miki et al., 1998a) acting downstream of tyrosine kinases and Cdc42. N-WASp is found in an inhibitory confirmation that is activated upon binding of PIP_2 and Cdc42,

releasing the VCA domain to interact with its cellular partners (Rohatgi et al., 2000), and this inhibited confirmation was subsequently also found to be the case with WASp (Kim et al., 2000). Over expression of N-WASp in a cell causes co-precipitation of N-WASp and actin in the cell cytoplasm (Miki et al., 1996) showing it has a potent actin polymerisation ability, more potent than WASp or WAVE in fact (Zalevsky et al., 2001), which is diminished when the WH2 domain is deleted (Yamaguchi et al., 2000; Yamaguchi et al., 2002). This actin polymerisation ability could be important in neural cells, where it is highly expressed, for the formation of neural growth cones (Banzai et al., 2000). In addition the intracellular pathogen *Shigella*, utilises N-WASp to move through the cytoplasm generating an actin tail to propel it along (Suzuki et al., 1998). There are no reported human cases of *N-WAS* deficient patients. Interestingly when a knockout mouse was made it was embryonic lethal suggesting that neither WASp nor WAVE could compensate for N-WASp. Therefore there may be different signalling events regulating the different proteins (Snapper et al., 2001).

1.4.2 Scar/WAVE Proteins

Originally discovered in *Dictyostelium* as Scar (suppressor of cAR) (Bear et al., 1998) but subsequently were also isolated as three mammalian homologues WAVE 1, 2 and 3 (WASp family Verprolin homologous protein) (Miki et al., 1998b; Suetsugu et al., 1999). They have a similar C-terminus to WASp but the N-terminus has a SCAR/WAVE homology domain rather than a Wiskott homology domain and do not possess a GTPase binding domain (**Figure 1.2**). Consequently, unlike WASp, they are not associated with Rho GTPases and may be regulated through different mechanisms. Mechanisms found so far include Abl interacting protein, which is involved in Ras to Rac signalling (Blagg and Insall, 2004).

Dominant negative mutants of WAVE1 lack Rac-dependant ruffling and neurite extension (Miki et al., 1998b). In Dictyostelium, loss of Scar leads to reduced F actin content and impaired cell motility and chemotaxis (Bear et al., 1998). WAVE1 knockout mice have severe brain impairments (Soderling et al., 2003) and WAVE2 knockouts are embryonic lethal (Yan et al., 2003), again showing little or no overlap of function with WASp or N-WASp.

1.4.3 Bee/1p

Bee/1p is the yeast homologue of WASp but lacks a GTPase binding domain (**Figure 1.2**). It has been shown to be a component of the cortical actin cytoskeleton in yeast, playing an essential role in the organisation of actin filaments. When Bee1p is knocked out there are defects in cytokinesis and budding (Li, 1997).

1.5 Cellular Defects of WASp

This section will discuss some of the cellular defects seen in patients with WASp, how they can be explained with respect of the protein structure and how the lack of a functional actin polymerisation response to a stimulus can lead to some of the clinical features seen.

1.5.1 T Cells

There is overwhelming evidence from the multiple defects seen in patient T cells that WASp is crucial for effective regulated T cell function. Patient T cells have been shown to be defective in their proliferative capability in response to various stimuli, including antigenic and allogenic (Ochs et al., 1980) and impaired IL-2 secretion upon

cross-linking of CD3ε (Molina et al., 1993). The lymphocytes are reduced in number (Ochs et al., 1980) and have been shown to exhibit morphological defects by having a reduced number of microvilli on the cell surface (Kenney et al., 1986), however recently this has been disputed (Majstoravich et al., 2004) and is possibly related to T cell activation states. There is a defect in receptor capping upon stimulation (Molina et al., 1993), which previously had been shown to be dependant upon actin polymerisation (Ryser et al., 1982). The immunological synapse is impaired without WASp, preventing optimisation of cell signalling, and therefore subsequent signalling events, such as cytokine secretion and proliferation (Badour et al., 2003a). Following TCR ligation WASp is recruited to lipid rafts that allow lateral movement of the TCR. In WAS patients and knockout mice there is a reduction in the number and upregulation of lipid rafts following T cell activation, preventing efficient responses to stimuli (Dupre et al., 2002). When WASp was re-introduced using a retroviral vector the lipid raft clustering and subsequent T cell activation was restored (Dupre et al., 2004).

T cell trafficking may also be impaired and contribute to the disease phenotype as the T cell chemoattractant SDF-1α has been shown to induce phosphorylation of WASp (Okabe et al., 2002) thus directing migration and diapedesis.

The defects shown in the T cells ability to respond to stimuli, ultimately due to an inability to polymerise actin, and therefore a lack in helper function, have been argued to be the main cause of immune dysregulation seen in WAS patients. This however usually reflects the specific interests of the investigators concerned, as other groups have identified major defects in other lineages e.g DC homing (de Noronha et al., 2005), that have also been theorised to be the main cause of the symptoms in WAS.

1.5.2 B Cells

B cells are not thought to be as severely affected in WAS as cells from other lineages. However B cells from WAS patients have been found to have defective transmembrane signalling (Simon et al., 1992) with reduced numbers of microvilli on the cell surface (Zhang et al., 1999). EBV transformed B lymphoblastic cell lines from patients have defective actin polymerisation and chemotaxis (Facchetti et al., 1998). Defects in migration may explain the aberrant splenic architecture seen in patients and mice (Vermi et al., 1999; de Noronha et al., 2005). The abnormal immunoglobulin levels seen in patients may in part be due to lack of T cell help as well as inherent problems with the B cells. Further evidence that WASp is not as important in B cells was shown in a patient that had somatically mutated a lymphoid progenitor so that both B and T cells expressed normal WASp. A selective advantage was seen in the T cell lineage but not the B cell lineage (Konno et al., 2004). This data supports that seen in murine knockout models (Snapper et al., 1998; Zhang et al., 1999) where T cells but not B cells are affected.

1.5.3 Natural Killer cells

As in T cells, NK cell activation relies on the formation of an immunological synapse and lipid raft clustering. Activation is reliant upon efficient actin polymerisation, as WASp and F-actin have been shown to be present in the immunological synapse of activated NK cells (Orange et al., 2002). In WAS patients although there are increased numbers of NK cells (Morio et al., 1989) their cytotoxicity is impaired (Gismondi et al., 2004). Introduction of IL-2 can correct the cytotoxicity (Gismondi et al., 2004) suggesting a lack of signal transduction is responsible for the defects, which is similar

to that observed in T cells. This lack of signalling and therefore cytotoxicity also probably partly explains the susceptibility of WAS patients to viral infections.

1.5.4 Platelets

Although thrombocytopaenia and small platelets are classical symptoms of WAS (Semple et al., 1997), the exact role of WASp in their development and function is still not known. *In vitro* experiments have shown WAS megakaryocytes to have abnormal cytoarchitecture, but platelets formed in this system were of a normal size (Haddad et al., 1999). Lack of WASp has been implicated in failure of maintenance of platelet integrity and aggregation at sites of haemorrhage with circulatory damage proposed as a reason for small platelets (Thrasher and Kinnon, 2000b). However WASp deficient platelets can activate Arp2/3 to form filopodia (Falet et al., 2002) making the reasons for the defects unclear.

1.5.5 Myeloid Cells

The ability of neutrophils, dendritic cells and monocytes to respond to a stimulus, whether through migration, efficient antigen presentation or through phagocytosis, is reliant upon quick and specific *de novo* actin polymerisation. As actin polymerisation has been shown to be defective in other cell types it would be reasonable to assume these myeloid cells, which rely heavily upon actin regulation for their function would also have defective actin polymerisation and therefore associated defects in their function. Specific defects of myeloid cells are discussed in more detail below.

1.5.5.1 Chemotaxis

Macrophages and dendritic cells have to be able to migrate through tissues when they are stimulated to go to a site of inflammation or to secondary lymphoid tissues to stimulate other cells of the immune system. Cell locomotion is controlled by protrusions of the cell membrane and is reliant upon spatial and temporal regulation of actin polymerisation. WAS macrophages have been shown to have abrogated chemotactic responses to fMLP, MCP-1 (Badolato et al., 1998), and CSF-1 (Zicha et al., 1998) compared to normal cells. Dendritic cells from WAS patients also have intrinsic defects in their actin regulation. When stimulated with fMLP or RANTES WAS DCs were non-motile. In addition, they did not polarise or extend dendritic processes (Binks et al., 1998).

Both macrophages (Linder et al., 1999) and DCs (Burns et al., 2001b) from *WAS* patients are lacking in specialised actin structures called podosomes. Podosomes are highly dynamic actin based structures with a half-life of 2-12 minutes that are in close contact with the substratum (**Figure 1.4**).

As can be seen in Figure 1.4, there is a basic actin core with actin regulatory proteins surrounded by a ring of vinculin and other cellular adhesion molecules (Marchisio et al., 1984). The regulation of podosome assembly and disassembly has been investigated in both macrophages and DCs and has been shown to be tightly regulated by WASp and Cdc42. They both associate in the podosome, and expression of the dominant negative form of Cdc42, V12Cdc42, in these cells leads to podosome disassembly (Burns et al., 2001b; Linder et al., 1999). Podosomes are thought to be involved in cell motility through adhesion to extra cellular matrix proteins and localised release of proteases allowing the cells to migrate through the substratum.

Figure 1.4 Podosome
Cartoon representation of a podosome present on the apical surface of a cell with structure components and associated proteins. The podosome is comprised of a central core of filamentous actin, Arp2/3 and WASp surrounded by adhesion molecules such as vinculin and integrins amongst others shown. Matrix metalloproteinases are thought to be secreted from the centre of the podosome (pink arrow). Figure adapted from (Linder and Kopp, 2005).

Release of MMPs has been shown in osteoclasts (Delaisse et al., 2000) and vascular smooth muscle cells (Burgstaller and Gimona, 2005). In fact WAS DCs do not adhere as strongly to ICAM-1 and do not assemble integrins around podosomes confirming podosomes are involved in adhesion (Burns et al., 2004). Chemotactic defects in macrophages and DC are therefore due to at least two defects. Firstly there is an abrogation in filopodia mediated by Cdc42 and WASp, shown to be crucial for chemotaxis (Allen et al., 1997; Allen et al., 1998). Secondly abnormal polarisation and adherence to substrata leads to an inability to direct movement in a regulated manner.

In addition to the chemotaxis defects *in vitro*, migration and homing defects of Langerhan cells have been demonstrated *in vivo* in a WAS KO mouse model (de Noronha et al., 2005). In deletion experiments, the minimal region of WASp required for chemotaxis is the VCA region. This is also the minimal region needed to stimulate actin nucleation, further linking the defects in *WAS* with dysregulation of actin polymerisation (Yarar et al., 1999). Figure 1.5 shows how the defects in WASp deficient dendritic cells affect a normal immune response.

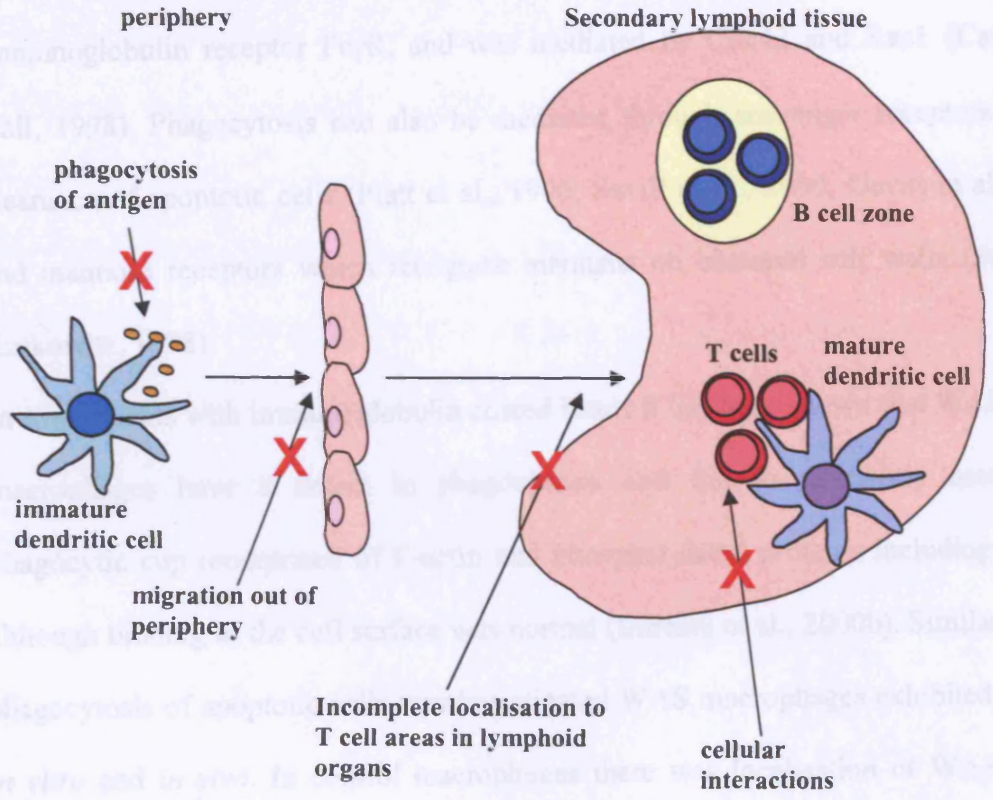


Figure 1.5 Defects in WASp deficient dendritic cells
WASp deficient dendritic cells fail to phagocytose some antigen and then show delayed migration from the periphery to secondary lymphoid organs. Upon arrival there is abnormal distribution within the T cell zones in secondary lymphoid tissue and failure to form an immunological synapse with T cells. (All deficiencies marked by an X).

1.5.5.2 Phagocytosis

In addition to chemotactic defects there have been several demonstrations of phagocytic defects in WAS patient macrophages. Phagocytosis is the active engulfment of particles by macrophages or DC. This can be in response to infections where bacteria are the target, virally infected cells, tumour cells for antigen presentation, or in the resolution of an inflammatory response where apoptotic cells are removed thus preventing chronic inflammation. Studies of phagocytosis have revealed two distinct mechanisms of phagocytosis involving different GTPases. One involving the complement receptor CR3 was mediated by Rho. The other mechanism was via the immunoglobulin receptor FcγR, and was mediated by Cdc42 and Rac1 (Caron and Hall, 1998). Phagocytosis can also be mediated through scavenger receptors for the clearance of apoptotic cells (Platt et al., 1996; Savill et al., 1990; Devitt et al., 1998) and mannose receptors which recognise mannans on bacterial cell walls (Stahl and Ezekowitz, 1998).

In experiments with immunoglobulin coated beads it has been shown that WAS patient macrophages have a defect in phagocytosis and fail to efficiently assemble a phagocytic cup (comprised of F-actin and phosphorylated proteins including WASp) although binding to the cell surface was normal (Lorenzi et al., 2000b). Similarly when phagocytosis of apoptotic cells was investigated WAS macrophages exhibited a defect *in vitro* and *in vivo*. In control macrophages there was localisation of WASp to the phagocytic cup supporting a role for WASp in phagocytosis (Leverrier et al., 2001b). So far there has been no association of CR3-mediated phagocytosis with WASp. As this is directed through Rho and there is little or no binding of Rho to WASp, this suggests that there is some redundancy where other WASp family members may play a role.

Another cell type of the myeloid lineage, the osteoclast, has been shown to be lacking in podosomes and does not form sealing zones, shown to be important in bone resorption, in WASp deficient mice (Calle et al., 2004). When WASp was reintroduced into these cells using a retrovirus, podosomes were restored along with normal cytoarchitecture (Calle et al., 2004).

These intrinsic defects in phagocytosis and chemotaxis in WAS macrophages and dendritic cells may contribute to the pathology seen in WAS. There is abnormal antigen presentation thereby adding to the immunodeficiency, and ineffective removal of EBV-driven lymphoproliferative disease increasing the susceptibility to autoimmune disease. A failure to remove apoptotic cells may also result in unchecked inflammatory responses and in C1q-deficient mice has been linked to systemic lupus erythematosus (Taylor et al., 2000). Thus it is possible that the increased autoimmunity seen in WAS patients is due to a breakdown of normal mechanisms of inflammatory suppression and maintenance of peripheral tolerance (Leverrier et al., 2001a). The abnormal dendritic cell migration to lymphoid organs in response to antigen may contribute to the immunodeficiency by not coming into contact with T cells in the correct environment and surveillance of the periphery for antigen or virally infected cells would therefore be impaired (Thrasher and Kinnon, 2000a).

Importantly when a normal copy of *WAS* is introduced into WASp null macrophages (Jones et al., 2002) and osteoclasts (Calle et al., 2004), podosomes are restored, as is chemotaxis showing the importance of WASp in these cytoskeletal defects. Additionally introduction of WASp into WAS null DCs leads to normal polarisation and podosome formation (Burns et al., 2001a). This is encouraging for gene therapy

protocols, as introduction of a functional copy of the gene leading to expression of WASp appears to correct the cellular cytoskeletal defects.

1.6 Murine Models of WASp

Generally murine models of human diseases are important to elucidate mechanisms of pathology and often mimic the human form of the disease. There are two murine models of Wiskott Aldrich syndrome based on Sv/129 backgrounds (Snapper et al., 1998; Zhang et al., 1999). Both are knockouts, caused by an insertion of a neo cassette in exon 7 and exon 4, respectively, resulting in a lack of protein expression and both reveal a reduction in the total number of lymphocytes in the periphery. However, the model by Zhang *et al.* reveals a defect in lymphocyte development (Zhang et al., 1999) not seen in the Snapper model. Both models reveal defects in T cell proliferation and receptor capping in response to CD3ε and have been used to elucidate the function of WASp in T cell responses. B cells in both models respond normally to antigen receptor stimulation with receptor capping and normal levels of proliferation. In addition they have normal levels of immunoglobulin.

Platelets in knockout mice are present in reduced numbers, although they are of normal size (murine platelets are smaller than human platelets), which mimics the defect seen in humans.

Mice in this study were kindly donated by S. Snapper. Dendritic cells and osteoclasts from the Snapper mouse model have a defect in the number of podosomes (Burns et al., 2001b; Calle et al., 2004) and exhibit defects in chemotaxis (Burns et al., 2001a) and homing (de Noronha et al., 2005) similar to that observed in human cells.

Haematopoietic stem cells isolated from knock out mice are defective in their migration compared to normal cells (Lacout et al., 2003) and this has been suggested to be the

mechanism through which skewing of X inactivation is observed. Stem cells expressing WASp can home to the bone marrow preferentially, thus the WASp null cells (which express from a mutant X) do not survive as well.

WASp mice do not develop eczema or haematological malignancies, although this may be related to age and maintenance in a pathogen free environment. They do however develop chronic colitis with lymphocyte infiltrations, which is not seen in humans, but is often seen in mouse models with defects in T cell function (Snapper et al., 1998).

In conclusion, WASp deficient mice are a relevant model for use in further explaining the defects seen in WAS and for further elucidating WASp function. This is emphasised in experiments where WASp has been introduced into WAS null cells and function has been restored (Dupre et al., 2004; Calle et al., 2004; Charrier et al., 2005).

1.7 Treatments for Wiskott-Aldrich Syndrome

If left untreated, patients with typical WAS have a poor prognosis and often die in early childhood. A variety of treatments have arisen in the years since WAS was first characterised, in an effort to improve the survival rate. Prophylactic antibiotics and immunoglobulins are given in an effort to reduce infections and therefore inflammation (Litzman et al., 1996). A splenectomy can be effective at increasing platelet numbers reducing the risk of haemorrhaging (Lum et al., 1980) as the platelets are not removed, however it does increase the risk of infection. This has increased the life expectancy of children but a significant proportion of patients still die before 10 years of age (Sullivan et al., 1994).

The only effective curative treatment so far is a bone marrow transplant (BMT). In a recent study of worldwide treatment, 61.8% of all centres and 95% of centres that treat 10 or more patients recommend a bone marrow transplant if a HLA matched sibling

donor is available. This is reduced to 55.2% and 77.7%, respectively, for matched unrelated donors (Conley et al., 2003). The survival rate for allogeneic transplants performed below the age of 5 years is 80% whereas above 5 years this fell below 50% (Thrasher and Kinnon, 2000b). In a study of WAS patients who had received a BMT some sustain full chimerism whereas others have mixed chimerism with a high proportion of WAS negative cells in the myeloid lineage and thrombocytopaenia. Due to the reduced survival rates without a matched donor, a lack of donors and complications arising from conditioning patients to accept the graft, alternative treatments are sought.

One alternative treatment to bone marrow transplantation is somatic gene therapy. With the increase in understanding of the function and regulation of WASp and improved gene therapy vectors, coupled with gene therapy successes for other primary immunodeficiencies, WAS is an attractive target for *ex vivo* stem cell gene therapy for several reasons. Firstly there is good characterisation of WASp expression and function, including murine models allowing safety and efficacy testing. Normally there is WASp expression from early in haematopoietic development, therefore there should be no abnormal reaction to WASp expression and it has been shown to be crucial in homing (Lacout et al., 2003) in the murine model. Somatic mutations have revealed a selective advantage, thus low expression and low transduction levels may not be a problem. These benefits are emphasised by the restoration of cytoskeletal actin structures and cell signalling when *WAS* is introduced into *WAS* null cells (Candotti et al., 1999; Wada et al., 2002; Burns et al., 2001b; Dupre et al., 2004; Calle et al., 2004; Strom et al., 2003b; Strom et al., 2003a; Charrier et al., 2005) and when normal cells are introduced into knock out mice (Strom et al., 2002; Charrier et al., 2005).

1.8 Stem Cells

Stem cells are defined as being pluripotent cells that are capable of self-renewing. They have been discovered deriving from various tissues e.g neural (Reynolds and Weiss, 1992) mesenchymal (Pittenger et al., 1999) and haematopoietic (Ogawa, 1993; Orlic and Bodine, 1994). Until recently, it was thought stem cells from one tissue could only give rise to cells of that tissue. Subsequently stem cells have been shown to be able to "trans-differentiate" into cells of tissues other than their origin through undefined re-programming pathways e.g. muscle to blood (Jackson et al., 1999) blood to liver (Lagasse et al., 2000) and blood to muscle (Ferrari et al., 1998) although this is still controversial.

1.8.1 Human Haematopoietic Stem Cells

Haematopoietic stem cells (HSC) can be derived from bone marrow, mobilised peripheral blood and umbilical cord blood and are able to give rise to all the cells in blood and the immune system, as outlined in Figure 1.6, and have been discovered in most vertebrate species, with human and mice being the most widely characterised. They have none of the lineage markers found on more mature cells, however various cell markers have been used to define human stem cells, with one of the first being CD34 (Strauss et al., 1991).

Experiments using murine xenograft models which allow human haematopoietic cell engraftment e.g. SCID, S/NOD, common γ-chain/RAG knock out, subsequently showed that the most primitive cells with the highest repopulating capacity are $CD34^+$ $CD38^-$ (Larochelle et al., 1996; Bhatia et al., 1997). The frequency of scid repopulating cells, and therefore pluripotency, varies depending on the source, with mobilised

peripheral blood having the lowest (1 in $6x10^6$ total cells), then bone marrow (1 in $3x10^6$) and finally cord blood (1 in $9.3x10^5$) having the highest (Wang et al., 1997). More recently CD133 (also known as AC133) has been shown to be a stem cell marker (Yin et al., 1997), as has CDCP1 (Cub-domain containing protein) (Conze et al., 2003) and KDR (Ziegler et al., 1999). Although these markers along with others have been found on stem cells they may not be definitive markers as their expression levels can change with cell cycle status.

1.8.2 Murine Stem Cells

Murine stem cells are also characterised by the lack of lineage markers (Spangrude et al., 1988) and the presence of cell surface markers such as Sca-1, thy-1 and c-kit, although expression levels can be strain dependant (Uchida and Weissman, 1992; Spangrude and Brooks, 1993). As has been observed in humans these populations are not pure stem cell populations with other cells expressing some of these markers. Although CD34 is expressed on foetal haematopoietic stem cells in mice by adulthood ~90% do not express CD34, with the $CD34^{-/low}$ subset of $Sca\text{-}1^+c\text{-}kit^+$ cells having a greater stem cell frequency (Osawa et al., 1996). So unlike humans, CD34 is not a marker for murine stem cells.

Figure 1.6 Development of the haematopoietic system
Pluripotent stem cells undergo division and commitment to multipotent progenitors or renewal. They in turn give rise to common lymphoid precursors (CLP) or common myeloid precursors (CMP). Further division and commitment lead to all the lymphoid and myeloid lineages of the blood. Granulocyte monocyte precursor (GMP), megakaryocyte erythrocyte precursor (MEP), erythrocyte progenitor (ErP), megakaryocyte progenitor (MkP). Adapted from (Reya et al., 2001)

1.8.3 Gene Transfer into HSC

Haematopoietic stem cells (HSC) are an attractive target for gene therapy because they are long lived and have a large capacity for self-renewal and, due to their pluripotency, if transduced would repopulate the blood with transduced progeny. In fact the plasticity of haematopoietic stem cells may allow correction of non-haematopoeitic tissues (Theise, 2004). They are easily accessible from different sources (bone marrow, cord blood and mobilised peripheral blood) with standard large-scale purification (typically immunomagnetic separation) and readily delivered back to patients by established transplantation techniques. However due to their quiescence and lack of receptors on their cell surface they are generally difficult to transduce.

The main focus of HSC gene transfer is to acheive high efficiency transduction whilst maintaining the repopulating ability and pluripotency. Many groups have focused on using cytokine cocktails that allow maintenance and even expansion of stem cells *ex vivo* without losing the pluripotency and engraftment potential as extended culture results in loss of the stem cell phenotype and homing potential (Blundell et al., 1999; Ahmed et al., 2004). Combinations of Stem cell factor (SCF), interleukins 3 and 6 (IL-3, IL-6), Flt-3 ligand (Flt-3L) and thrombopoeitin (Tpo) have been used successfully to culture and expand primitive HSC in humans (Bhatia et al., 1997; Conneally et al., 1997b; Conneally et al., 1998; Demaison et al., 2000; Cavazzana-Calvo et al., 2000a; Gaspar et al., 2004), and mice (Moreau-Gaudry et al., 2001; Strom et al., 2003b; Klein et al., 2003; Uchida et al., 2003) whilst maintaining engraftment potential enabling *ex vivo* transduction to take place.

In addition to cytokine stimulation other methods of maintaining the self-renewal properties of HSC have been investigated by deconstruction and manipulation of the

molecular pathways normally regulated in HSC (reviewed in (Stein et al., 2004)). These include transcription factors such as homeobox genes e.g. HoxB4 which is expressed in primitive but not lineage specific cells. When HoxB4 was over expressed in murine HSC it lead to a repopulation advantage over untransduced cells whilst still able to fully reconstitute the HSC compartment (Thorsteinsdottir et al., 1999). Other pathways looked at include cell cycle regulators. Entry into cell cycle in stem cells is governed by the cyclin-dependant kinase inhibitors p21 and p27, and when they are absent in mice there is an increase in the stem cell pool size (Cheng et al., 2000; Cheng et al., 2001). It is hoped that further understanding of the molecular pathways through which stem cell commitment and self-renewal is regulated may allow transduction *ex vivo* whilst maintaining pluripotency.

1.9 Gene Therapy

Gene therapy is the complementation or replacement of a defective copy of a gene by the addition and expression of a correct copy, in *trans,* via recombinant DNA technology thus restoring a normal phenotype.

Somatic gene therapy has been used as a therapeutic tool for over ten years and although originally developed for single-gene defects (Kay and Woo, 1994), it has now been developed for a wide range of diseases such as cardiovascular disease (Isner, 2002), neurodegenerative disorders (Baekelandt et al., 2000), infectious diseases (Bunnell and Morgan, 1998) and cancer therapies (Kay et al., 2001). In fact cancer therapies make up the majority of all gene therapy clinical trials, comprising over 63% with varied efforts to fight cancer, approaches include delivering anti-angiogenic factors, tumour suppressor genes and immunostimulatory genes (Mccormick, 2001).

Generally, gene therapy strategies would require high levels of transduction of the target cells, that is sustained at therapeutic levels as long as is needed without the need for repeat procedures. These criteria have been tackled in many different ways with different vectors comprising of two main types. Firstly there are vectors derived from viruses where the virus has been modified through removal of genes involved in replication, toxicity and immunogenicity to become safe and to allow packaging and delivery of the transgene ready for expression in the target cell.

The other types of vectors are synthetic and termed non-viral. After initial experiments with naked DNA they now encompass a multitude of different approaches to deliver DNA efficiently and safely. Both approaches have their advantages and disadvantages for different diseases and are summarised in table 1.1.

1.9.1 Viral Vectors

Viruses are highly evolved to infect cells, and to deliver and replicate a genetic package using the host cell machinery. Viral vectors for gene therapy utilise the efficient infection pathways but are replication defective and have reduced toxicity. Many different viruses have been adapted to make them into vectors suitable for gene therapy. These include adenovirus, adeno-associated virus, herpes simplex virus, gamma retroviruses and lentiviruses (**Table 1.1**).

Table 1.1 Properties of Commonly used Gene Transfer vectors (adapted from (Nathwani et al., 2005)

1.9.2 Requirements for stem cell gene therapy

Due to the inefficiency of non-viral vectors a viral vector is preferred. Ideally the viral vector most suited for use in correction of immunodeficiencies by gene therapy would be one with a good tropism for haematopoietic stem cells. Efficient infection and therefore delivery of the transgene would make use of the current techniques in stem cell culture without losing the pluripotent potential of the HSC. Additionally integration of the transgene into the host chromosome would be beneficial for long-term gene expression and for correction of all lineages. The viral vectors would ideally be non-immunogenic, as an immune response could hinder the efficacy of the treatment as well as be harmful to the patient. Finally the vector would need to be able to carry a relatively large sized transgene, in the order of 9kb, to enable most single gene genetic defects to be treated and the vector should be easily prepared with large scale preparation a necessity for a clinical trial.

As can be seen in Table 1.1, although adenovirus and herpes simplex virus (HSV) can package DNA constructs of large length and can infect non-dividing cells, they do not have good tropism for HSC (Wickham et al., 1993) and do not integrate into the host genome. Additionally even though they have had many immunogenic genes removed they can still elicit host immune responses. Adeno-associated virus (AAV) can only package a small transgene, has low integration and poor tropism for HSC. There is a high incidence of host antibodies to AAV although no symptoms or disease is known to result from AAV infection. Utilisation of alternative serotypes may overcome these problems however (Nathwani et al., 2005).

Retroviral vectors, including simple type C and complex lentiviruses, can infect HSC and integrate their DNA (once reverse transcribed) into the host genome. They can package relatively large transgenes and are non-immunogenic. There are packaging cell

lines available enabling easy preparation and scale up for clinical trials. Therefore gamma retroviral and lentiviral vectors may be the best suited for use in the treatment of single gene defect immunodeficiencies.

1.9.3 Retroviruses

Retroviruses are enveloped viruses consisting of 2 copies of single stranded RNA and are so named due to their ability to transcribe RNA to DNA. The main feature promoting them to the forefront of viral gene therapy vectors is their ability to integrate into the host genome. They can be classified simply into three groups: spumaviruses e.g. foamy virus, lentiviruses e.g. HIV-1, with all the others classed as onco-retroviruses e.g. Murine leukaemia virus.

All retroviruses contain at least three coding domains: *gag*, *pol* and *env* within long terminal repeats (LTR) responsible for integration of the provirus into the host genome. The LTR also has promoter and enhancer functions thus regulating the expression of the viral proteins. *gag* encodes for the matrix, capsid and nucleoproteins; *pol* encodes the viral protease (*pro*), reverse transcriptase and integrase and finally *env* encodes the envelope glycoprotein. Complex retroviruses such as lentivirus and spumavirus have additional accessory genes that encode regulatory elements effecting transcription, infectivity/virulence and RNA splicing.

1.9.4 Retrovirus Life cycle

A simplified model of the infection of a cell followed by budding of new viral progeny is shown in Figure 1.7. The virus particle binds to the cell via a cell surface receptor and this initiates the entry of the virus through fusion of the two membranes resulting in

the insertion of the viral core. The single stranded RNA is reverse transcribed to double stranded DNA and is transported to the nucleus. Entry to the nucleus allows the proviral DNA to integrate into the host genome. Translation by the host cellular RNA polymerase II allows transcription of spliced mRNA, for expression of viral proteins, and of full length RNA for packaging into new virus particles. New virus particles are assembled at the cell membrane and new infectious virions are released by budding from the host membrane.

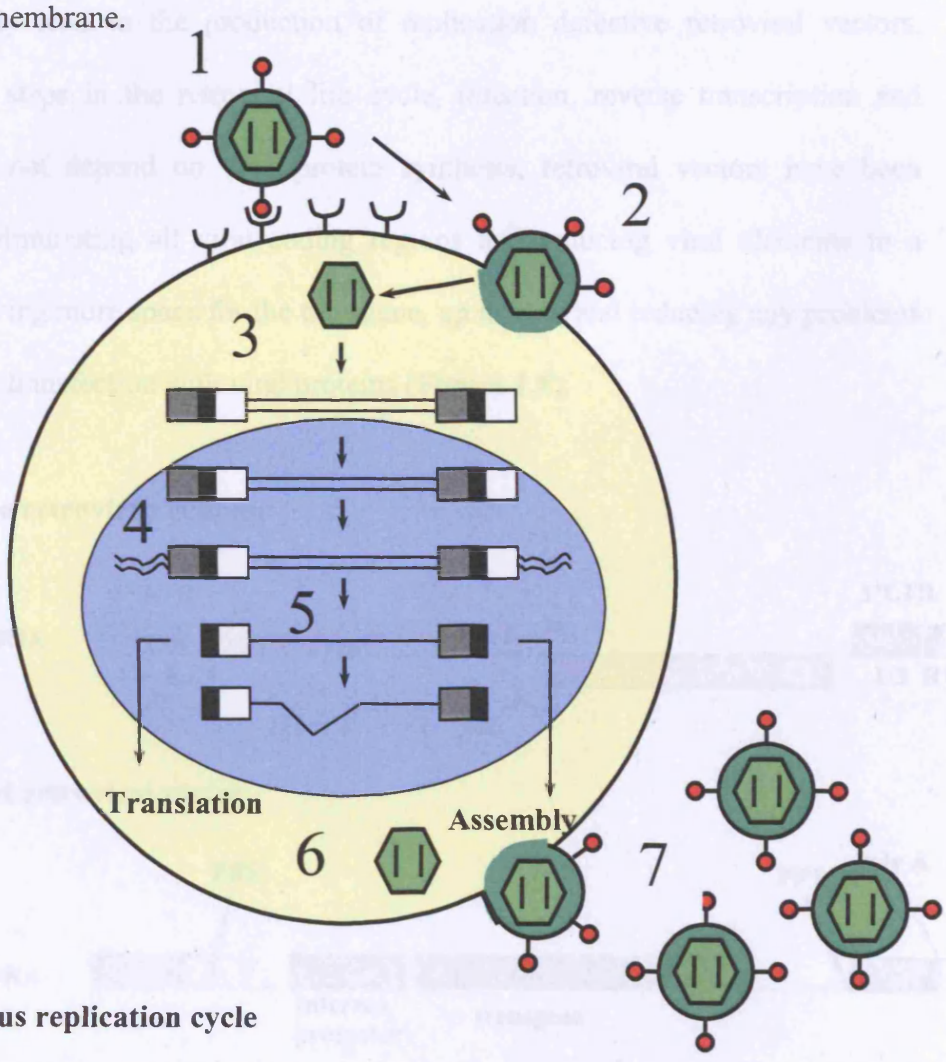


Figure 1.7 Retrovirus replication cycle

1. The parental virus attaches to a specific receptor on the surface of a permissive cell. **2**. The virion and cell membrane fuse leading to entry of the core. **3**. The RNA is reverse transcribed to generate a double stranded DNA copy. **4**. The provirus DNA is transported to the nucleus and integrated into host chromosomal DNA. **5**. Translation by host cell RNA polymerase II generates RNA copies of full length and spliced messenger RNAs. **6**. Messenger RNA is translated in the cytoplasm to form viral proteins. These are assembled together at the plasma membrane with full length RNA to form progeny virus. **7**. Budding releases progeny virus able to infect other cells.

1.9.5 Principles of Retroviral Vector Design

The basic principles of retroviral design involve making the virus safe and replication defective whilst maintaining their ability to efficiently transduce target cells. Synthetic retroviral vectors were first produced in the early 1980s using replication-competent helper virus but this was not acceptable, as the replication competent virus could not be separated. The development of packaging cell lines that provided retroviral proteins in *trans* eventually lead to the production of replication defective retroviral vectors. Because early steps in the retroviral life cycle, infection, reverse transcription and integration do not depend on viral protein synthesis, retroviral vectors have been improved by eliminating all viral coding regions and reducing viral elements to a minimum allowing more space for the transgene, up to 9kb, and reducing any problems associated with transfection with viral proteins (**Figure 1.8**).

Figure 1.8 Development of type C Retroviral Vectors

1/. Proviral genome structure of murine leukaemia virus (size 8.8kb). **2/.** Genome structure of a MLV based viral vector. Gag, pro, pol and env have been removed and replaced with the transgene but the essential packaging sequence (ψ), primer binding site (PBS), poly purine tract (PPT) and poly adenylation sites are retained. An internal promoter is optional if there are is an intact U3 region on the 3' LTR but necessary in SIN vectors where the U3 region has been deleted. The transgene and promoter can be in either orientation between the viral LTRs. Adapted from (Hu and Pathak, 2000)

Most of the vectors used in gene transfer are derived from mammalian type-C viruses such as Moloney murine leukaemia virus (MMLV), although other types have been used. The simplest vectors have a transgene with the expression controlled through the viral LTR and a packaging signal to incorporate the viral RNA correctly within the virion. More complex vectors have internal promoters, alternative splicing, multiple promoters and internal ribosomal entry sites to enable multiple gene expression often including selectable markers. An additional safety feature is the deletion of the U3 region of the 3' LTR. This deletion is transferred to the 5' LTR during reverse transcription making transcription after integration impossible without an internal promoter, but maintains the poly A signal and PPT. Use of tissue or cell specific promoters can therefore provide some regulation of the transgene expression when compared to the ubiquitous LTR driven expression. This will be discussed in more detail later.

1.9.6 Packaging Cell Lines

Various packaging cell lines have been developed from adherent cell lines such as NIH3T3, 293T and HT1080, which provide the helper function necessary for production of replication deficient virus. They constitutively express the virus packaging genes on separate plasmids, to reduce the probability of recombination, under the control of drug resistance genes to maintain expression. Some packaging cell lines e.g. the 293T based Phoenix cell line used in this study also express markers which are linked to *gag*, *pol* and *env* expression thus allowing selection of high expressers and ultimately high titer virus producing cells (Kinsella and Nolan, 1996; Grignani et al., 1998b). Without the addition of the transfer vector packaging cells secrete empty particles, but upon transfection with a transfer vector, virus particles

containing the transgene are secreted (**figure 1.9**). As vector design has improved so have packaging cell lines. There are many different packaging cell lines available including those that are pseudotyped with different envelopes compared to the original virus. This allows the tropism of the virus to be broadened and to increase the efficiency of transduction in cells that are lacking in certain viral receptors. An ecotropic envelope packaging cell line allows production of virus able to infect mouse cells only. The receptor is a cationic amino acid transporter, CAT-1 (Albritton et al., 1989). For production of virus able to infect both human and mouse there is the amphotropic envelope whose receptor is Pit 2, a phosphate transporter (Kavanaugh et al., 1994; Miller et al., 1994). Other popular envelopes pseudotyped with MLV based retroviruses are Gibbon-Ape Leukaemia Virus (GALV) which binds Pit 1, another phosphate transporter (Kavanaugh et al., 1994; Miller et al., 1994), RD114 which binds RDR, a neutral amino acid transporter (Rasko et al., 1999) and Vesicular Stomatitis Virus-G (VSV-G) which binds the cell membrane component phosphatidylserine (Mastromarino et al., 1987).

Virus with the ecotropic envelope is able to infect murine stem cells, as there are high levels of the receptor on the cell surface. The amphotropic receptor is present at lower levels on both murine and human stem cells, therefore resulting in low transduction efficiency. Improved transduction efficiency in these cells has been seen with GALV and RD114 pseudotyped vectors, when compared to amphotropic and this has been directly linked to increased amounts of receptor (Thomsen et al., 1998).

Furthermore the RD114 and VSV-G envelopes stabilise the virus particles. This allows concentration of the virus by centrifugation, allowing for higher titers, and therefore higher multiplicity of infection leading to improved transduction efficiency (Gatlin et al., 2001).

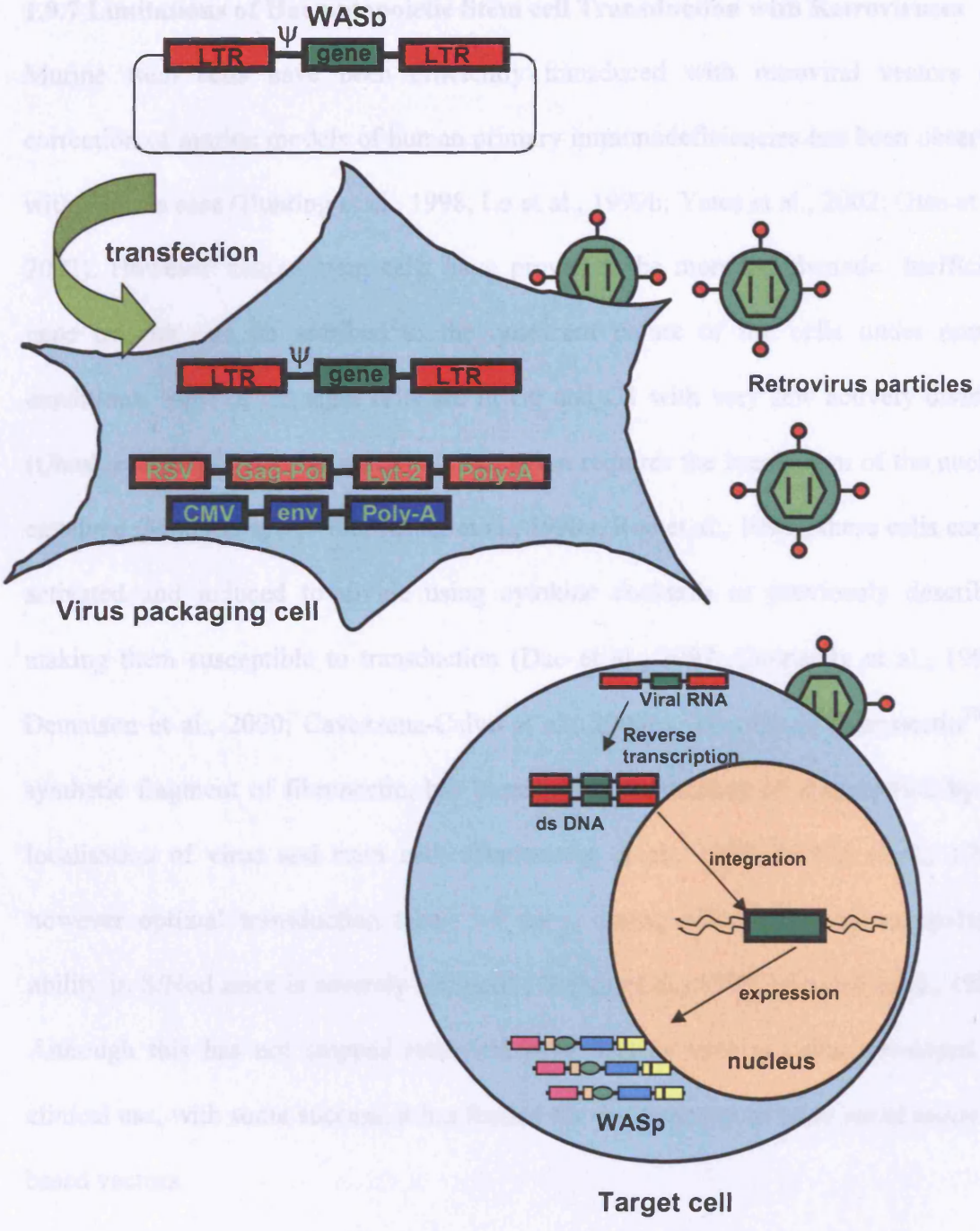


Figure 1.9 Retroviral packaging cell lines
The transfer vector containing the gene of interest (in this case WASp) in between 2 LTRs and a packaging signal (ψ) is transfected into a packaging cell stably transfected with Gag-Pol and Env to provide all elements *in trans* necessary for formation of intact viral particles (Pheonix cells are the packaging cells shown). The packaging cell can have various Env genes to enable pseudotyping of the virus particle. Upon infection of the target cell the viral RNA is reverse transcribed to double stranded DNA and integrated randomly into the host genome. The strong expression from the viral LTRs gives rise to cellular expression of the transgene.

1.9.7 Limitations of Haematopoietic Stem cell Transduction with Retroviruses

Murine stem cells have been efficiently transduced with retroviral vectors and correction of murine models of human primary immunodeficiencies has been observed with relative ease (Bunting et al., 1998; Lo et al., 1999b; Yates et al., 2002; Otsu et al., 2002). However human stem cells have proved to be more problematic. Inefficient gene transfer can be ascribed to the quiescent nature of the cells under normal conditions. Most of the stem cells are in G0 and G1 with very few actively dividing (Cheshier et al., 1999). As retroviral integration requires the breakdown of the nuclear envelope (Miller et al., 1990b; Miller et al., 1990a; Roe et al., 1993), these cells can be activated and induced to divide using cytokine cocktails as previously described, making them susceptible to transduction (Dao et al., 1997; Conneally et al., 1997a; Demaison et al., 2000; Cavazzana-Calvo et al., 2000b). The use of Retronectin™, a synthetic fragment of fibronectin, has increased the efficiency of transduction by co-localisation of virus and stem cells (Hanenberg et al., 1996; Moritz et al., 1996), however optimal transduction takes 3-4 days, during which time the repopulating ability in S/Nod mice is severely impaired (Gothot et al., 1998; Blundell et al., 1999). Although this has not stopped retroviral gene therapy vectors being developed for clinical use, with some success, it has fuelled the development of other novel retrovirus based vectors.

1.9.8 HIV-1 Lentivirus

The lentivirus is a complex retrovirus consisting of six additional genes to the previously described three for simple retroviruses (**Figure 1.10**), however they do not have oncogenic potential. They have an advantage over simple retroviruses in that they can infect non-dividing cells (Lewis and Emerman, 1994). Vectors for use in gene

therapy are mainly derived from HIV-1 (Naldini et al., 1996), but have also been made from HIV-2 (D'Costa et al., 2001), SIV (Schnell et al., 2000) and EIAV (Yamada et al., 2001). The most widely used and most advanced complex retrovirus based vectors are HIV-1 derived.

In addition to *gag, pol*, and *env* found in simple retroviruses, HIV-1 has four accessory proteins; *vif, vpu, vpr* and *nef*, and two regulatory elements; *tat* and *rev*. The accessory proteins found in complex retroviruses enhance the efficiency of the viral life cycle such as mRNA production, protein production and transport to and from the nucleus, but unfortunately they also add to the virulence and pathology associated with the virus. In wild type virus vpr is required for replication of the virus and targeting virus to the nucleus in non dividing cells via its nuclear localisation sequence (Heinzinger et al., 1994b). In addition it can induce cell cycle arrest in infected cells (Trono, 1995). vif is important for the production of highly infectious mature virions through unknown mechanisms (Gabuzda et al., 1992). *vpu* encodes for a small integral membrane protein. In T cells it acts upon CD4 accelerating its destruction in the endoplasmic reticulum (ER) therefore down regulating it on the cell surface. The subsequent release of env from CD4 binding in the ER promotes transport of env to the cell surface and enhances release of virus from the cell membrane (Willey et al., 1992; Gottlinger et al., 1993). The product of the *nef* gene is membrane associated, has complex effects on signal transduction within the cell and it too down regulates CD4, but from the cell surface (Greenberg et al., 1997). nef also has an effect on reverse transcription as virus particles without *nef* are less infective and have reduced viral DNA synthesis (Schwartz et al., 1995).

Figure 1.10 Development of HIV-1 derived gene transfer vectors

1/. Wildtype HIV-1 genome showing the full-length proviral DNA, the open reading frames (ORFs) and viral proteins. **2/.** First-, **3/.** Second-, **4/.** Third generation HIV-1 based lentiviral vectors showing the progressive loss of viral genes and the replacement of the LTRs with a CMV promoter and the addition of a poly A signal. All the structural and accessory proteins and maintained in the first generation vectors but are lost in the second generation. The third generation further splits rev onto another plasmid for increased safety. **5/.** The transfer vector contains the gene of interest flanked between the LTRs. The 3' LTR is U3 deleted, which is duplicated during reverse transcription, for increased safety but therefore requires an internal promoter. Additional features include a central poly purine tract and the woodchuck post-transcriptional response element. **6/.** The envelope plasmid allows pseudotyping viral particles with the VSV-G envelope, altering the tropism compared to wildtype. (Adapted from (Brenner and Malech, 2003).

Finally the remaining two genes *tat* and *rev* are responsible for activating transcription and regulating splicing and RNA transport respectively. tat binds to a region in the viral LTR called TAR (Tat activation region) and is a potent activator of HIV-1 gene expression from the viral LTR (Laspia et al., 1989). rev binds to a cis-acting RNA target, the Rev Responsive Element (RRE) (**Figure 1.10**), present in all unspliced viral transcripts and targets them for nuclear export thus allowing alternative splicing necessary to generate the array of mRNAs required for expression of all viral genes (reviewed in (Pollard and Malim, 1998)). These two genes are necessary for virus replication, whereas the other four are associated with virulence.

The infection and replication cycle of HIV-1 is similar to that of simple retroviruses in that once the virus has entered the cell there is reverse transcription of the RNA to DNA followed by integration and translation. The subsequent differences in transcriptional activation and regulation have been discussed, as have the effects of the virulence factors. The main difference driving vector development is the ability to infect non-dividing cells. This is by actively transporting the pre-integration complex through the nuclear membrane pore via a nuclear localization signal in an energy dependant manner (Bukrinsky et al., 1992) even though the pre-integration complex is larger than the nuclear pores. The pre-integration complex is comprised of nucleic acid, the matrix protein p17, reverse transcriptase, integrase and vpr (**Figure 1.11**). The matrix protein and vpr can be removed without loss of integration as vectors have been made without either suggesting some redundancy (Heinzinger et al., 1994a). However a non-classical nuclear localisation signal has been found in the catalytic domain of integrase and seems to be critical for transport across the nuclear membrane (Bouyac-Bertoia et al., 2001). In addition, subsequent studies have revealed the triple stranded

DNA flap created by reverse transcription of the central polypurine tract (cPPT) is important for nuclear localisation (Zennou et al., 2000).

Critically for development of lentiviral vectors the pathogenic features need to be removed and the risk of generation of replication competent virus eliminated whilst maintaining the specific feature of transduction of non-dividing cells.

Figure 1.11 The lentiviral pre-integration complex.
The viral cDNA, complete with DNA flap, forms a complex with Vpr, matrix protein p17 (MA) integrase (IN) and reverse transcriptase (RT) in the cell cytoplasm. This complex is transported through the nuclear pore into the nucleus where the cDNA is able to integrate into the host genome. Adapted from (Sherman and Greene, 2002)

1.9.9 Development of Lentiviral Vectors

The development of lentiviral vectors can be grouped into 'generations' where each subsequent generation reveals a major change and/or improvement from the last (**figure 1.10**), although this nomenclature is not specifically defined in the literature.

The first generation of vectors consisted of breaking the viral genome into three plasmids similar to the process first developed for retroviruses (Naldini Science 1996), to reduce the chance of a replication competent virus being produced. The three plasmids produced were: a transfer vector containing the 'gene of interest' (either a marker or therapeutic gene) located in-between the viral LTRs, a packaging construct containing the helper functions and the envelope containing construct. The helper or packaging construct (pCMVΔ8.9) contains *gag* and *pol* with all the accessory virulence factors apart from *vpu* but has no LTR sequences or packaging signal. A CMV promoter controls expression in place of the 5'LTR and a poly A signal derived from the insulin gene replaces the 3'LTR (Naldini et al., 1996). The transfer vector (pHR') containing the viral LTRs has most of the viral *gag* and *pol* sequences removed, as is the *env* and all of the accessory genes, whilst retaining the rev and tat responsive elements (RRE and TAR). The RRE allows complex expression elements including introns and genomic sequences to be included by taking advantage of the nuclear export function of rev. Removal of the RRE results in a dramatic reduction in the titer. With the removal of most of the wild-type genome, up to 10kb can be inserted between the LTRs. The third plasmid contains the *env* gene under the control of the CMV promoter. HIV-1 normally has the narrow tropism of macrophages and T cells, but will tolerate pseudotyping. Therefore the original envelopes used were either the VSV-G envelope, which confers a

broad based tropism due to its ability to bind to phosphatidylserines in the plasma membrane or the MLV amphotropic envelope.

Virus is made by transiently transfecting all three plasmids into an adherent cell line such as 293T cells and harvesting the supernatant 48-72 hours later. VSV-G pseudotyped particles can be concentrated by ultracentrifugation due to their stability (Naldini et al., 1996). This allows the production of replication defective infectious virus that can integrate into cells not undergoing mitosis. Unfortunately there are few HIV-1 packaging cell lines possibly due to the toxicity associated with the VSV-G envelope (Klages et al., 2000) or HIV-1 protease (Ikeda et al., 2003).

Second generation vectors have further changes in the packaging plasmid. The accessory genes (*vif, vpu, vpr, nef*) are removed with the regulatory elements *tat* and *rev* retained. The efficiency of virus production was not affected by the extra deletions however there was a reduction in efficiency of transduction in some cell types e.g. macrophages (Zufferey et al., 1997). This was thought to be due to dependence upon *vpr* and *vif* and is cell type dependant, as irradiated 293T cells did not have a reduced infectivity. Further modifications include the manipulation of the 3'LTR by partially deleting the U3 region. This creates a self-inactivating (SIN) vector due to both U3 regions of the cDNA created from the 3'LTR during reverse transcription. Deletion of the U3 region eliminates transcriptional activity of the viral LTR, and prevents synthesis of viral RNA, therefore an internal promoter was required (Zufferey et al., 1998). The creation of SIN vectors improved the safety of the vectors by minimizing the risk of creating replication competent virus and abnormal expression of cellular genes mediated by the viral LTR. In addition the removal of the enhancer sequences prevents distant promoters being activated and interference between specific promoters and the viral LTRs (Zufferey et al., 1998).

Third generation vectors further split and deleted the lentivirus genome for additional safety, whilst maintaining the titers obtained and the transduction efficiencies. *tat* was made redundant, and therefore removed, by the placement of a constitutive promoter in place of the U3 portion of the 5'LTR. *rev* was separated from the helper plasmid onto a new non-overlapping construct under the control of an RSV promoter (Dull et al., 1998). This created a four plasmid system available for use in a similar manner to the three plasmid system. These additional safety features render the plasmids non-functional outside of the producer cells and further decreases the likelihood of recombination events leading to replication competent viral particles being produced whilst removing as much of the wild type HIV-1 genome as possible.

Enhanced expression from SIN vectors has been achieved through the incorporation of the central polypurine tract (cPPT) and the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE). The cPPT acts by enhancing nuclear transport and increasing the amount of integrated vector DNA promoting higher transduction (Follenzi et al., 2000) especially in stem cells (Sirven et al., 2000). The inclusion of the WPRE enhances transport of vector RNA from the nucleus (Schambach et al., 2000) and increases the efficiency of transduction (Zufferey et al., 1999).

1.10 Regulation of Gene Expression

Historically one of the main problems associated with gene transfer, especially into haematopoietic cells, is long term maintenance of high levels of expression of the transgene. Many vectors utilise the CMV promoter which works well *in vitro* but can often be silenced *in vivo* by *de novo* methylation (Challita and Kohn, 1994a). This has led to the use of other promoters such as phosphoglycerate kinase (pgk) (Zufferey et al.,

1998; Follenzi et al., 2000) and elongation factor 1α (EF1α) (Salmon et al., 2000). Use of different retroviral 3' LTR sequences such as those from the spleen focus forming virus (SFFV) have also shown increased levels of expression in haematopoietic lineages (Baum et al., 1995) and with the WPRE and cPPT have vastly improved the expression levels in haematopoietic cells (Demaison et al., 2002).

Although high transgene expression is often required there may be some problems associated. Dissemination of particles, although not a problem *ex vivo*, could lead to transduction of non-target cells *in vivo* raising the possibility of germ-line transmission. Over expression of transgenes and expression in cells that do not normally express a protein may lead to adverse effects. Therefore the regulation of transgene expression through inducible promoters or tissue specific promoters has been investigated with the aims of improving the efficiency, safety and duration of expression possibly regulated within the cell in a normal manner.

Regulation of gene expression by inducible promoters has been achieved by systems such as the tet on/off system (Vigna et al., 2002) or ecdysone (Galimi et al., 2005). This allows regulation of the amount of protein expressed, cessation of expression and repeated treatment cycles if needed. These systems are however reliant upon the easy administration of the inducer/repressor and reliable activation leading to expression of the transgene.

Tissue specific promoters would allow promiscuous infection whilst stopping unwanted expression in undesirable cell types. They have been described for brain (Hwang et al., 2001), muscle (Li et al., 1999), bone (Hou et al., 1999), and liver (Nash et al., 2004). In addition to tissue specific expression, cell or lineage restricted expression has also been investigated. These include T cells (Indraccolo et al., 2001), macrophages (Gough and Raines, 2003) megakaryocytes (Yasui et al., 2005) and red blood cells (May et al.,

2000) amongst others. These promoters whilst maintaining specificity often do not give expression levels comparable to constitutively active promoters and therefore are not always optimal.

Critically primary immunodeficiencies such as WAS often have defects in multiple lineages therefore a haematopoietic specific promoter would be required. There is some evidence to suggest that the putative promoter sequence 3' of the start codon of WAS shows some haematopoietic restriction (Petrella et al., 1998; Martin et al., 2005) thus making it an attractive promoter for use in retroviral or lentiviral gene therapy protocols.

1.11 Statement of Aims

The aims of this study were:

- To develop a fusion protein of EGFP and WASp to elucidate the role of WASp in cells using direct fluorescence and for use in gene therapy protocols.
- To restore WASp expression to therapeutic levels in a WAS KO mouse model by transducing bone marrow derived stem cells using this fusion protein in a retrovirus
- Establish the efficiency of HIV-1 based lentiviral vectors encoding EGFP-WASp and WASp to restore functional activity in murine dendritic cells and human WASp null macrophages, using a podosome based assay. This includes a comparison of internal promoters including constitutive, derived from viruses, and tissue specific, derived from the region immediately 5' of the *WAS* start codon to determine the best efficacy.

- Further comparison of the HIV-1 based vectors ability to restore WASp expression, and therefore phenotype *in vivo,* by introduction of WASp in a WASp KO mouse model.
- Investigate constitutively active $WASp^{I294T}$ in patient primary cells and induced expression in a cell line to determine possible mechanisms of patient phenotype.

CHAPTER 2

MATERIALS AND METHODS

All reagents from Sigma, unless otherwise stated. All primers from Invitrogen.

2.1 Cell Culture

2.1.1 Cell Lines

293T cells, Phoenix Eco, HeLa cells, HT1080 cells and NIH 3T3 cells were purchased from the European Collection of Cell Cultures (ECCC) and cultured in Dulbecco's MEM- Glutamax (Life Technologies) supplemented with 10% FCS (Sigma) 100IU/ml penicillin and 100ug/ml streptomycin (Life Technologies).

Jurkat cells and Cos-7 cells were purchased from ECCC and were maintained in RPMI-glutamax supplemented with 10% FCS and 100IU/ml penicillin and 100ug/ml streptomycin (complete). All the cultures were maintained at 37°C with 5% CO_2 and split every 3-4 days when approaching confluency. Adherent cells were detached by incubating with trypsin-EDTA for 5-10 min prior to splitting.

Bac-1 macrophages (a gift from G Jones, Kings College, London) were cultured in complete RPMI supplemented with 30ng/ml of rhuM-CSF were split every 3-4 days. Incubation with Accutase at 37°C for 5-10 min was used to detach adherent cells.

2.1.2 Human Peripheral Blood Macrophages

10ml human blood from patient or a normal control was venisected into heparinised tubes, diluted 1:1 with RPMI and layered onto Ficol-Hypaque (Pharmacia, Sweden) and centrifuged at 2300rpm, without brake, for 20 min. The peripheral blood mononuclear cells were aspirated from the ficol/serum interface and washed in RPMI (Life

Technologies) twice. These cells were then plated into 25cm^2 flasks in complete RPMI and incubated at 37°C for 2 hours to allow the monocytes to adhere. After 2 hours the non-adherent cells were removed and fresh complete RPMI was added supplemented with 10ng/ml rHu-MCSF (R+D Systems, Oxon, UK). The media was refreshed every 2 days until day 5 or 6 when the macrophages were harvested for use. Purity was determined by CD14+ staining.

2.1.3 Murine Dendritic Cell Culture

Bone marrow was obtained by flushing the femur and tibia of mice with RPMI. The single cell suspension was plated into 75cm^2 flasks in HEPES-buffered RPMI Glutamax-1 supplemented with 10% FCS, 100IU/ml penicillin and 100ug/ml streptomycin, and 25ng/ml rmuGM-CSF (Peprotech). After 48 hours the media was replaced with complete RPMI containing 25ng/ml rmuGM-CSF and 10ng/ml rmuIL-4 (Peprotech). The cells were further grown for 72-96 hours prior to the non-adherent cells being harvested for use. Purity was determined by CD11c+ staining.

2.1.4 Purification of murine stem cells

Bone marrow was flushed from the femur and tibia of WASp KO or control Sv/129 mice with RPMI and washed in PBS/1%BSA (w/v). The sca1+ve cells were either positively selected using MACS sca1 microbeads (Miltennyi Biotech) or lineage negative cells obtained by negative selection to enrich progenitor cells (Stem Cell Technologies).

For positive selection, 100ul of MACS beads were added to 1x10^8 cells in 400ul PBS/1%BSA and incubated at 4°C for 20 minutes. Unbound beads were diluted out

with 10mls of PBS/1%BSA and the cells pelleted before resuspension in 1ml of PBS/1%BSA. A MACS MS column was equilibrated with 1ml PBS/1%BSA and the cells applied to the column. The column was washed with 1ml PBS/1%BSA before the bound cells were released by adding 1ml RPMI 30% FCS (v/v) and forcing off the column using the syringe plunger. The cells were counted, an aliquot tested for purity by flow cytometric analysis of the Sca1+ population, and cultured at concentrations of $5x10^5$-$1x10^6$ in 100ng/ml SCF, 10ng/ml Flt-3L and 20ng/ml IL-6 (Peprotech).

For negative selection cells were resuspended at 2-$8x10^7$/ml in PBS 1%BSA. Cells were then incubated with rat serum (final concentration 5%) at 4°C for 15 minutes, followed by addition of the antibody cocktail at the indicated concentration and further incubation for 15 minutes at 4°C. Cells were washed and 100ul/ml of anti-biotin tetrameric complexes was added, incubated at 4°C for 15 minutes and finally 60ul/ml of magnetic colloid added followed by a further 4°C incubation for 15 minutes. The sample was loaded onto a prepared column and the enriched progenitors collected as flow through. Cells were counted and cultured as described previously for Sca1+ selected cells.

2.2 Immunostaining of Cells

2.2.1 Human dendritic cells, macrophages and murine dendritic cells

On day 6, 10^4 macrophages or dendritic cells were transferred to 13mm diameter glass cover slips coated with fibronectin, where stated, and allowed to adhere. Prior to immunostaining, the cells were fixed in 4% paraformaldehyde (PFA), permeabilised with 0.1% Triton X-100 and blocked with PBS/1% BSA for 30 minutes. Cells were incubated with rhodamine phalloidin (Molecular Probes, Leiden, Netherlands) for 20

min to visualise F-actin and then with anti-vinculin antibody (hVIN-1 mouse IgG1 monoclonal, ascitic fluid used at 1:100 dilution). Antibody binding was detected with a goat anti-mouse IgG conjugated to Cy-5 (Jackson Immunoresearch, West Grove, PA, USA). Images were recorded digitally using either a charge coupled device (CCD) camera or a confocal Leica (TCS-SP2) microscope to obtain composite images or projections of 8-12 z sections. Images were processed with Adobe Photoshop®.

2.2.2 Adherent Cell Lines

Cells were plated onto 13mm diameter or 22mm^2 coverslips at densities between 10^3-10^5 in their respective media, allowed to adhere overnight, before fixing and staining as described previously.

2.2.3 Reconstituted Mice

Murine bone marrow, axial and brachial lymph nodes, spleen and thymus were excised from mice and a single cell suspension made in PBS/1%BSA. 1x10^6 cells were washed twice in PBS/1%BSA before incubating with antibody at 1:100 dilution against CD3, CD11b or B220 at 4°C for 20 minutes. The cells were washed twice in PBS/1%BSA and once in PBS before fixing in 4% PFA.

2.2.4 Cell Cycle analysis

HT1080 cells were grown as previously described. At intervals between days 2-15 cells samples were harvested, washed in PBS and fixed in PFA overnight. After 24 hours they were washed with PBS and incubated on ice in 70% ethanol for 2-3 hours. The cells were washed again and resuspended in PI buffer, (1mg/ml Na3 Citrate, 0.1% triton), PI added at 50ug/ml and analysed by flow cytometry within 15 minutes for

DNA and *EGFP* levels. The data was collected using Expo32 software (Beckman Coulter). Doublet discrimination was used to include only single cells. 100,000 events were collected to permit cell cycle analysis of both the GFP-negative and GFP-positive fractions. Linear gates were set to quantify cells at each phase of the cell cycle. Additional analysis was performed using Multicycle software (Phoenix Flow Systems). The aggregate model was applied to the data to exclude doublets and a curve fitted to model DNA distribution within the cell cycle.

2.2.5 Apoptosis and Cell Death Analysis

HT1080 cells were maintained as previously described and at intervals between day 2-28 samples were harvested, washed in PBS and resuspended in annexin buffer prior to staining for annexin V PE (Pharmingen) and 7AAD to determine the levels of apoptosis and cell death. The cells were analysed within 20 minutes of staining and 20,000 cells collected to allow both the GFP positive and negative fraction to be analysed.

2.3 Virus Production

2.3.1 Ecotropic and Amphotropic Retrovirus

Phoenix retrovirus producer cells containing either the ecotropic (a gift from Garry Nolan), were plated at 70% confluency and transfected with PINCO constructs containing EGFP, EGFP-WASp or WASp by the calcium phosphate precipitation method. The cells were treated with puromycin at 1ug/ml for 1-2 weeks until a highly selected population was obtained. The cells were removed from selection washed and plated at high confluency with just enough media to cover (5ml in 75cm^2 flask) and the

supernatant containing the virus harvested twice daily for 2-3 days. Virus was stored at –80°C.

2.3.2 Lentivirus Production by Ultracentrifugation

Viral stocks were produced by co-transfecting 3 plasmids into 10-15x10^6 293T cells: the envelope plasmid pMD.G (10ug), the helper plasmid pCMVR8.91 (30ug) and one of the transfer vectors encoding EGFP, EGFP-WASp or vector containing WASp (40ug) After 48 hours the resulting viral supernatant was concentrated by ultracentrifugation at 100,000g in a Beckman centrifuge to pellet the virus, followed by resuspension in RPMI or Stemspan (Stem Cell Technologies) and stored at –80°C until used.

2.4 Viral infection of cells

2.4.1 Retrovirus Titering

50,000 3T3 cells were seeded in dilutions of viral supernatant from 1:1 to 1:250. After 72 hours the cells were harvested and analysed by flow cytometry to determine the percent positive cells. The titer was then be calculated by multiplying the number of cells seeded by the dilution and the percent of positive cells.

2.4.2 Lentivirus Titering by Infectious method

50,000 HeLa cells were seeded in dilutions of viral supernatant from 1:100 to 1:10^6. After 72 hours the cells were harvested as previously described and the titer calculated as before.

2.4.3 Lentiviral Titering by Quantitative pcr

2.4.3.1 Purification of genomic DNA from Cultured cells

Cultured cells were pelleted and 20ul of NP40 lysis buffer containing proteinase K was added. This was incubated at 56°C for 2 hours, briefly pulsed at 13,000 rpm, before further incubation for 15minutes at 95°C to inactivate the proteinase K. The solution was again pulsed at 13,000rpm and 180ul of distilled DNAse free water added. Following centrifugation at 13,000rpm for 10 minutes the supernatant was collected and transferred to another tube. This was either used immediately or frozen at -20°C until needed.

50,000 HT1080 cells were seeded and dilutions of viral supernatant added as described previously. After 72 hours the cells were harvested, pelleted and genomic DNA purified from the cell pellet. Two separate PCR reactions were run for each sample in triplicate using a fluorescently labelled probe (ABI) against the lentiviral vector primer binding site and against β actin. The probes are fluorescent labelled on the 5' end and have a quencher on the 3' terminus. The endonuclease activity of *taq* releases the 5' fluorescent label from the fret activity of the quencher thus increasing the fluorescence in the well with each cycle of the pcr, which can be detected. This would allow a value to be obtained for the number of viral copies present and the number of cell genomes in the reaction. By comparing the cycle threshold values, an arbitrary value where all the samples are in log phase expansion and are increasing without converging, obtained against a standard curve for the lentiviral vector and β actin, the number of copies of integrated virus could be calculated per cell. The viral titer can then be calculated by multiplying the integrated copy number by the number of cells plated and the dilution of virus added initially.

2.4.3.2 Creation of standard curves

A standard curve of known copies of lentiviral plasmid was generated from 1 copy to 100,000 copies/5ul added to the PCR reaction. The number of lentiviral vector genomes was calculated using the following formulae.

Plasmid size (bp) x 330 Daltons x 2nt/bp = No of Daltons or g/mole

e.g. for SEWW. **11371 x 660 = 7504860**

g/mole ÷ Avagadro's constant = g/molecule

e.g. **$7504860 \div 6.023 \times 10^{23} = 1.246 \times 10^{-17}$**

Therefore, 8.9ug/ml of plasmid (actual concentration) has

$0.0089 g/ml \div 1.246 \times 10^{-17} = 7.1429 \times 10^{14}$ molecules/ml

The plasmid DNA can then be diluted to the appropriate concentration per 5ul.

A plasmid was generated containing β actin and dilutions were made in a similar manner and normalised against known dilutions of cells (a gift from D King and S Howe, ICH).

2.4.4 p24 ELISA

The quantity of p24 in viral supernatants was calculated using a commercially available HIV-1 p24 antigen ELISA (Beckman Coulter). The assay was performed as per instructions. Briefly viral supernatant was diluted $1:1 \times 10^6$ and $1:5 \times 10^6$ and added to the antibody pre-coated wells. The samples were lysed and biotinylated human anti HIV-1 IgG added followed by incubation at 37°C. After washing a streptavidin horseradish peroxidase is added followed by tetramethylbenzidrine (TMB) and hydrogen peroxide to form a blue colour. This reaction is stopped by addition of acid and the intensity of the colour developed is directly proportional to the amount of HIV-1 in the sample. This can then be calibrated against p24 antigen standards at known dilutions.

2.4.5 Viral Infection of Cell Lines

Retroviral infection of cell lines was performed by seeding cells at a low confluency to induce cell division followed by addition of viral supernatant diluted 1:1 with fresh media. Multiple rounds of infection were performed to increase transduction levels if needed.

For lentiviral infection the concentrated virus was directly added to the cells in culture at the required MOI in small culture volumes with no conditioning.

2.4.6 Lentiviral Infection of Macrophages and Dendritic cells

Macrophages and dendritic cells were prepared as described previously and transferred to 13mm diameter coverslips, coated with fibronectin where stated. After 2-3 hours in culture to allow adherence lentivirus was added at the required MOI. The media was refreshed 24h later and the cells incubated for a further 48h before fixing and staining as previously described. Podosome restoration was determined by counting random fields of view captured as images by confocal microscopy (Leica). Images were manipulated in Adobe Photoshop® and 100-200 cells were scored for each condition. These images were further used to determine the number of podosomes per cell.

2.4.7 Retrovirus Infection of Murine Stem Cells For Reconstitution

Sca1 positive cells were harvested as previously described placed into culture in 30% FCS RPMI (v/v) supplemented with 100ng/ml SCF, 20ng/ml IL-6 and 10ng/ml Flt-3L for 48 hours. Non-tissue culture plates were coated with retronectin (Takara) and virus supernatant was preloaded by centrifugation at 4000g for 20 minutes. Cultured Sca1+ cells were added to the retronectin coated wells and more viral supernatant added

diluted 1:1 with fresh media supplemented with cytokines. The cells underwent 4 further rounds of infection over the next 72 hours. Finally after 5 days the cells were washed and either plated out in semi-solid media as previously described or re-infused into lethally irradiated (1000 RADS) recipients at doses of 2-4x10^5/mouse.

2.4.8 Lentivirus Infection of Murine Stem Cells for Reconstitution

Sca1 positive cells or lineage negative cells were harvested and plated out as previously described overnight in media containing cytokines with the addition of the lentiviral supernatant at the stated MOI at an optimised concentration of 10^7-10^8/ml. After 24 hours the cells were washed and infused into lethally irradiated recipients at doses of 2-3x10^5/mouse.

2.5 Cloning

2.5.1 Restriction Endonucleases

1-4ug of DNA was digested for 1-2 hours with excess enzyme present. All digests were performed in 40-50ul in the optimal buffer and temperature recommended by the manufacturer (Promega or NEB unless stated).

2.5.2 Blunting 5' ends

After digestion if needed the DNA was blunted by addition of DNA polymerise I (Klenow Fragment) and 2mm dNTPs buffered as required. This was performed at room temperature for 15 minutes before heat inactivating the enzyme at 65°C for 15 minutes.

2.5.3 DNA cleaning

Digested and blunted DNA was cleaned using a Qiagen Nucleotide Clean up Kit. This removed all salts and previously added enzymes which could inhibit further manipulation.

2.5.4 Dephosphorylating DNA

Where required DNA was dephosphorylated using shrimp alkaline phosphatase. This required incubating the DNA with 1ul of enzyme in the supplied buffer at 37°C for 1 hour before heat inactivating for 15 minutes at 65°C.

2.5.5 Resolving digested DNA

After manipulation the DNA was run on a 1% agarose gel for 1-2 hours depending on the size of fragment to be resolved. The band was excised and purified from the agarose using a gel extraction kit (Qiagen)

2.5.6 Ligation

Suitably compatible digested and purified DNA fragments were mixed with DNA ligase and ligase buffer as per the manufacturers instructions and incubated overnight at 14°C.

2.5.7 Preparation of Chemically Competent Bacteria

50ml LB was inoculated with JM109 *E.coli* and incubated at 33°C with agitation for 4-8 hours. At regular intervals a sample was tested to determine the optical density at 650nm. When the density was between 0.6-0.7 the culture was spun down at 5000rpm for 15 minutes at 4°C and the resulting pellet resuspended in 10ml sterile 2M $CaCl_2$ for

15 minutes. The bacteria were spun again and resuspended in 4ml of fresh 2M $CaCl_2$ 1ml of sterile glycerol added and snap frozen in aliquots of 100ul in liquid nitrogen.

2.5.8 Transformation of Competent Bacteria

20-30ul of ligated DNA was incubated on ice with 100ul of competent bacteria for 30 minutes. The DNA bacteria mix was then incubated at 42°C for 90 seconds before returning to ice for 5 minutes. Then 1ml of LB without antibiotics was then added to the transformed bacteria and incubated at 33°C for 1hour. After incubation the bacteria were pelleted and spread onto an LB agar plate with the appropriate antibiotic selection and incubated overnight at 33°C until colonies derived from single bacteria could be observed.

2.5.9 Mini-preps

5-10ml LB containing the appropriate antibiotic (either 50ug/ml kanamycin or 100ug/ml ampicillin) was inoculated from a single colony and incubated overnight. Multiple colonies were picked and the resulting cultures were pelleted and the plasmid DNA extracted using a mini-prep kit (Qiagen). This DNA was digested to confirm the presence of the correct plasmid DNA construct within the bacteria.

2.5.10 Large Scale Plasmid DNA Production

500ml LB with the appropriate antibiotic was inoculated 1/1000 and incubated for 12-14 hours at 33°C with agitation. The bacteria were pelleted by centrifugation at 5000g for 15 minutes and the plasmid DNA extracted by alkaline lysis using a Mega-Prep Kit (Qiagen). The resulting DNA was resuspended in 1ml water and stored at –20°C.

2.6 Immunoblot

Cells from culture or primary cells were harvested and pelleted at 1200rpm for 5 minutes. They were then resuspended in 200ul of lysis buffer containing protease inhibitors and incubated on ice for 5 minutes. The sample was then spun at 14000rpm for 5 minutes at 4°C and 200ul of the supernatant added to 200ul of 2 X SDS loading buffer. This was then heated at 85°C for 5 minutes before loading onto gel or stored at –20°C until required.

Glass plates were cleaned and a gel poured. The plates were filled to ¾ full with a separating gel of 8% and allowed to set. The remaining ¼ was filled with a stacking gel of 12% (see appendix) and allowed to set. The gel was loaded with all wells filled in order to get straight running and a voltage of 150-250 volts applied for 1-3 hours depending on the size of the gel. The gel was transferred to nitrocellulose using a semi dry blotter at 12 volts for 20 minutes.

Before probing with antibodies the nitrocellulose was incubated for ½ hr-1hr in a 5% milk solution in PBS-T to block non-specific binding. The nitrocellulose was probed with a solution of antibody in 5% milk PBS-T for 1-2 hours before being washed 5 X in PBS-T. A secondary antibody conjugated to HRP was added for ½ -¾ hour in 5% milk solution in PBS-T, then the blot was again washed in PBS-T. Protein bands were visualised by incubating the nitrocellulose with ECL for 1 minute and exposing on photographic film or digitally captured using a gel documentation system (UVITEC) and software (UVISOFT).

Where necessary the nitrocellulose had the bound antibody stripped off by washing in 0.2M glycine, 0.5M NaCl for 5-10 minutes before rinsing in 0.1M NaOH for 10 minutes. The nitrocellulose could then be re-probed for β-actin to enable loading of the gel to be checked.

2.7 Quantitative PCR

2.7.1 Determination of Viral Copy Number in Transduced murine cells

Quantitative pcr was used to determine integrated viral copy number and has been described previously (Towers et al., 1999). The transduced murine cells were pelleted and genomic DNA extracted as previously described. Two separate pcr reactions were run in triplicate for each sample using a fluorescently labelled probe against the lentiviral vector primer binding site and against murine *titin* (a gift from A Galy, Genethon, Paris, France(Charrier et al., 2005)). This would allow a value to be obtained for the number of viral copies present and the number of murine cell genomes in the reaction. By comparing the cycle threshold values obtained against a standard curve for the lentiviral vector and murine *titin*, the number of copies of integrated virus could be calculated per cell. Standard curves were created by diluting plasmid as previously described. The value for murine *titin* was normalised for copy number per cell. After obtaining a value for copy number the value was normalised to that of another laboratory (A.Galy, Genethon, France) performing the same pcr on the same sample.

2.7.2.1 Extracting mRNA from cell pellets

Cell pellets were resuspended in Tri-reagent and $1/5^{th}$ total volume of chloroform (stabilised with amylenes) was added. This was centrifuged at 13,000rpm in a microfuge to separate the RNA, DNA and protein into specific layers. The clear top layer containing RNA was removed and an equal volume of isopropanol added. This was incubated at -20°C for at least 2 hours before centrifugation at 13,000rpm to pellet the precipitated RNA. The RNA pellet was washed in 70% ethanol, spun at 13,000rpm

for 5 minutes and air dried. The dry pellet was resuspended in 10-20ul of water and stored at -20°C until needed.

2.7.2.2 Making cDNA from RNA

5ul of RNA was incubated with 0.5ul RNAse inhibitor (Applied Biosystems), 1ul of an oligo dT and 1ul of 2.5mM each dNTPs at 70°C for 10 minutes followed by placing on ice for 5 minutes. 1ul of 25mM MgCl2, 1ul of pcr buffer 2 (Applied Biosystems) and 0.5ul MMLV reverse transcriptase (Applied Biosystems) was added and the mixture incubated at 42°C for 1 hour to allow reverse transcription. The resulting cDNA/mRNA hybrid could then be used in a pcr to determine mRNA levels in the cells.

2.7.2.3 *WAS* mRNA expression analysis

Two separate pcr reactions were performed in triplicate using the cDNA/mRNA hybrid as template to determine the expression levels of *WAS (*Assay by Demand, ABI). These were a human *WAS* specific pcr and a murine *PGK* pcr. By directly comparing the cycle threshold (CT) value obtained from the *WAS* pcr relative to the housekeeping gene the expression levels of human *WAS* can be compared across samples using one sample as the reference to which all others can be compared. The fold increase or decrease in expression levels are calculated in the following way.

WAS CT – _PGK_ CT = ΔCT for each sample

ΔCT sample – ΔCT reference = ΔΔCT

$2^{-\Delta\Delta CT}$ = fold increase between samples

2.8 T Cell proliferation Assay

Murine splenocytes, ($2x10^5$ cells/well in 200ul), were stimulated in triplicate for 72 hours in DMEM with 10%FCS with anti-CD3ε antibody (Pharmingen) coated onto 96 well flat bottomed microtiter plates. Cultures were pulsed with [methyl-^{3}H] thymidine (1uCi/well) for the last 16 hours. Incorporated radioactivity was measured using a scintillation counter.

2.9 Patient Leukocyte Functional assays

2.9.1 E.Coli Phagocytosis (Kit phagotest Orpegen)

100ul whole blood was added to separate FACS tubes containing 20ul FITC labeled opsonised e.coli ($1x10^9$ bacteria/ml). A control tube was kept a 4^0C, the test tube was incubated at 37^0C for 10 mins. The tubes were then put on ice and 100ul quench solution added to strip the FITC labeled bacteria which are outside of the cell. 2ml PBS was added and the tubes centrifuged at 1000xg for 5 mins, the supernatant was discarded and 1ml lysing solution added at room temp for 10 mins to remove erythrocytes. The tubes were centrifuged at 1000xg for 5 mins, cells resuspended in PBS and recentrifuged. Once the supernatant had been dicarded the cells were resuspended in 300ul PBS with 100ul DNA staining solution. The samples were kept on ice and analysed within 20 mins.

2.9.2 Oxidative Burst Response in Granulocytes (Kit phagoburst Orpegen)

100ul whole blood was added to separate FACS tubes containing 20ul PBS, 20ul of unlabelled opsonised e.coli ($1x10^9$ bacteria/ml), 20ul 5uM PMA solution, or 20ul 400nmol/l fMLP for which the blood has been preincubated for 3 minutes with 3ug/ml

cytochalasin B. The tubes were incubated for 10 mins at 37^0C, 20ul working solution of DHR is added, and the tubes were returned to 37^0C for a further 10 mins.
1ml lysing solution was added at room temp for 10 mins to remove erythrocytes. Then the tubes were centrifuged at 1000xg for 5 mins, the supernatant discarded, cells resuspended in PBS and recentrifuged. Once the supernatant had been dicarded the cells were resuspended in 300ul PBS with 100ul DNA staining solution. The samples were kept on ice and analysed within 20 mins.

2.9.3 Flow cytometry

All samples were analysed on a Beckman Coulter XL flow cytometer using Expo2 software. A live gate was enabled on the DNA stain (675nm) to eliminate debris and bacteria. 10,000 events were collected in the granulocyte gate identified by forward scatter side scatter analysis. Oxidative burst was observed using the FL1 (525nm) signal to detect the oxidation of DHR from a non fluorescent to fluorescent compound to evaluate the percentage and MFI above the PBS control response. Phagocytosis was observed using the FL1 (525nm) signal to detect the percentage of cells which had phagocytosed FITC labelled bacteria compared to the 4^0C control. The MFI was evaluated as a guide to the amount of bacteria within each cell.

2.9.4 Apoptosis in Patient Lymphocytes and Bone Marrow

Apoptosis in lymphocytes was measured by incubating the mononuclear cell fraction from density-centrifugation separated peripheral blood for 48 hours in RPMI / 10% FCS containing IL-2 (60IU/ml) and PHA (10μg/ml). For the induction of apoptosis, cells were transferred to a 48 well plate (1×10^5 cells/ml). Apoptosis restricted to the fas pathway was induced by the addition of 1μg/ml IgM anti CD95 antibody (CH11 clone

Kamiya Biomedical, Seattle, WA). Non specific cell death was induced by 0.1μg/ml camptothecin. A negative control, medium only, was included to assess spontaneous cell death. After a further 48 hour culture, cells were washed and resuspended in annexin buffer (Pharmingen, Oxford, UK) prior to staining with annexin-V FITC and 7AAD/propidium iodide (PI) to determine apoptosis and death levels. 10000 cells were read by Beckman Coulter XL FACS within 30 minutes of staining (Vermes et al., 1995; van Engeland et al., 1998). Spontaneous apoptosis in bone marrow was measured by incubating density-centrifugation separated cells for 15 minutes at room temperature with Annexin V FITC and PI to a final concentration of 1μg/ml for each. The signal was then quenched in Annexin buffer and analysed immediately. Cells had been pre-labelled by incubation with one of CD34, CD33 or CD15 for 30 minutes and then washed with PBS. Samples were analysed on the flow cytometer as previously described.

CHAPTER 3

ONCORETROVIRAL GENE THERAPY FOR WISKOTT-ALDRICH SYNDROME

3.1 Introduction

In order to elucidate the functional role of WASp in cells it is necessary to have an easy and reliable method of detection. A lack of suitable antibodies against WASp has required solutions such as FLAG tagging (Symons et al., 1996). Unfortunately this requires fixation of the cells and tagging on the conformationally sensitive C-terminus. Tagging the N-terminus with EGFP to create a fusion of EGFP and WASp was considered a viable option to detect WASp in live cells and to determine where in the cell WASp is located. Use of fluorescence is also useful for determination of transduction efficiencies, promoter activity and protein levels.

Viral somatic gene therapy for primary immunodeficiencies such as Wiskott-Aldrich syndrome would be best achieved using retroviral vectors due to the ease of virus production, transduction of cells and insertion of the transgene as has been discussed earlier. In fact retroviral gene therapy has been very successful in clinical trials involving X-linked SCID (Cavazzana-Calvo et al., 2000a; Hacein-Bey-Abina et al., 2002; Gaspar et al., 2004), clearing a pathway for other primary immunodeficiency diseases to be tackled.

The aims of this chapter were to firstly to make a fusion of EGFP and human WASp with evaluation to determine function *in vitro*. EGFP and EGFP-WASp were then cloned into an oncoretroviral vector for use in an *in vivo* gene therapy protocol to reconstitute a WASp KO murine model (Snapper et al., 1998).

3.2 Results

3.2.1 Creation of an EGFP-WASp fusion cDNA construct

WASp has a natural auto inhibited conformation which is released upon binding of PIP_2 and the GTP form of Cdc42 (Higgs and Pollard, 2000; Kim et al., 2000). Any large additions to the C-terminus to make a fusion could theoretically inhibit this inactive conformation and subsequently alter the properties. Previous attempts to detect WASp have been by tagging the C-terminus with small markers, which can then be detected with antibodies (Symons et al., 1996). As EGFP has a size of 25kDa, it may also be too large to add to the C-terminus without altering the properties of WASp. It was decided therefore to create a fusion protein where EGFP was added to the less conformationally active N-terminus of WAS thus theoretically allowing normal protein folding and thus normal regulation of activation upon stimulation.

The complete *WAS* cDNA was excised from FL3/41.5RI:0.5RI (a gift from J.Derry) (appendix 1) with SacII and EcoRV and ligated, in frame, into pEGFP-C2 (Clontech) (appendix 2) which had been linearised with SacII and Sma-I before the stop codon (**Figure 3.1a**). This created an EGFP-WASp fusion open reading frame with the two proteins sequences separated by 75bp due to the continued presence of the multiple cloning sites from the pEGFP-C2 vector. For ease of further sub-cloning a 60bp fragment between the two proteins containing the multiple cloning site, was removed by digestion with Xma III followed by re-ligation of the fragments (appendix 3). The open reading frame was maintained whilst removing the multiple cloning sites between the two protein sequences (**Figure 3.1b**). Digestion with EcoRI followed by separation of the DNA fragments on an agarose gel confirmed the cloning as *WAS* contains extra recognition sequences compared to EGFP (not shown).

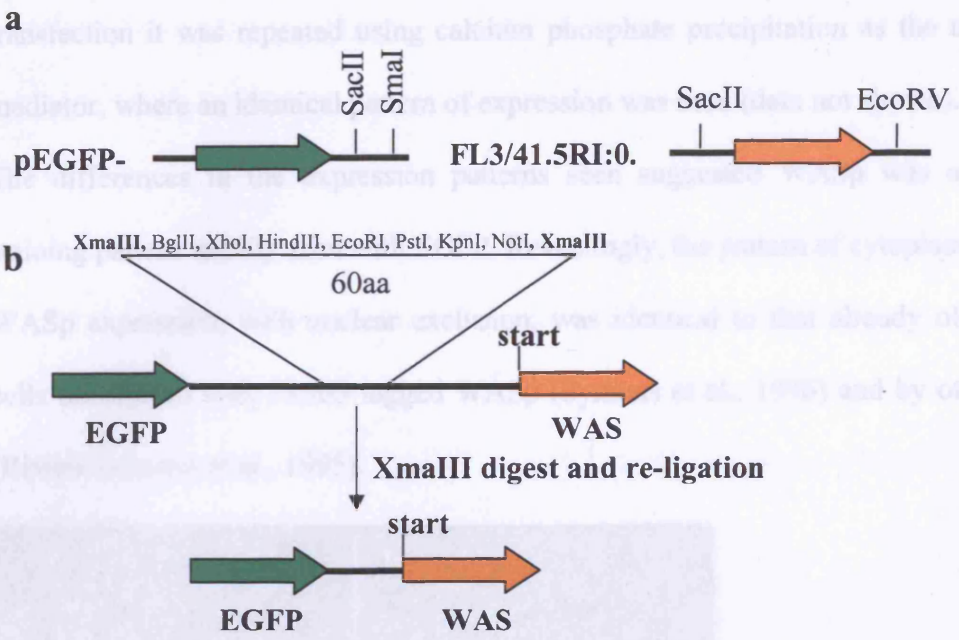


Figure 3.1 Creation of EGFP-WASp fusion
a) Plasmid maps showing position of restriction enzymes used to create fusion. **b)** Superfluous cDNA containing a MCS was removed by digestion with XmaIII whilst maintaining the fusion protein in frame.

3.2.1.1 Transient transfection of COS-7 cells

Confirmation that a fluorescent protein could be expressed and that it was a fusion of EGFP and WASp was next determined by transfecting a readily available cell line. COS-7 cells were seeded onto coverslips and transiently transfected with 1ug of DNA per well using an integrin binding peptide and lipofectin technique (LID) (Hart et al., 1997) at a ratio of 1:4:0.75, of DNA:peptide:lipofectin. The pattern of fluorescence 48 hours post transfection observed when cells were transfected with EGFP is diffuse and present throughout the cell (**Figure 3.2a,b**). EGFP-WASp transduced cells revealed a markedly different pattern of expression (**Figure 3.2c,d**). There was predominantly expression in the cytoplasm with exclusion from the nucleus. Expression even seemed to be sequestered to form aggregates within the cytoplasm. To confirm the expression pattern was the result of expression of a fusion protein and not an artefact of

transfection it was repeated using calcium phosphate precipitation as the transfection mediator, where an identical pattern of expression was seen (data not shown).

The differences in the expression patterns seen suggested WASp was altering the staining pattern usually seen with EGFP. Revealingly, the pattern of cytoplasmic EGFP-WASp expression, with nuclear exclusion, was identical to that already observed for cells transfected with FLAG tagged WASp (Symons et al., 1996) and by other groups (Rivero-Lezcano et al., 1995).

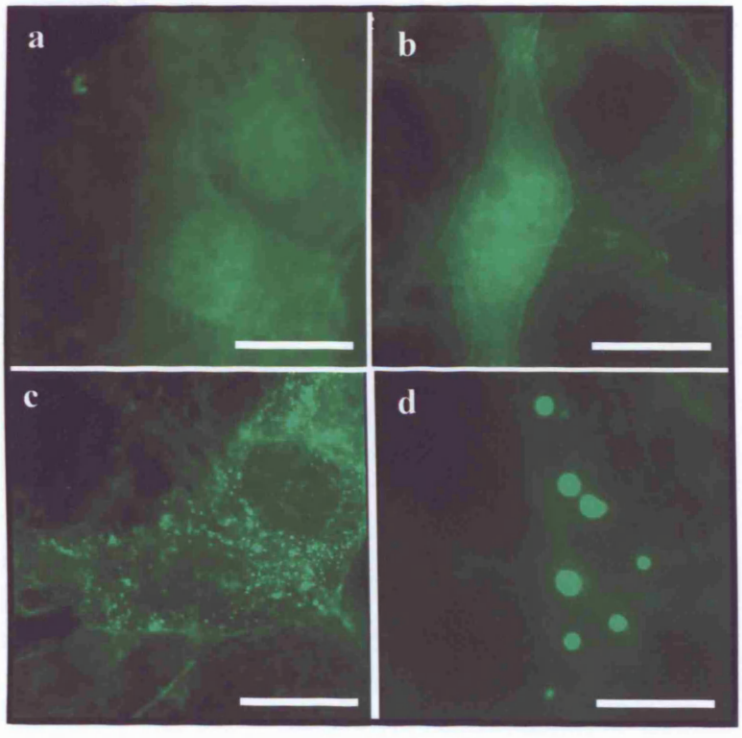


Figure 3.2 CCD images of transfected COS-7 cells
a+b) pEGFP and c+d) pEGFP-WASp transiently transfected into COS-7 cells using lipid and integrin binding peptide. Cells were fixed 48 hours post transfection and images captured using a CCD digital camera. Scale bar = 10um

3.2.1.2 Confirmation of expression of a fusion protein

To confirm there was expression of a fusion of EGFP and WASp an immunoblot was performed on transfected 293T cells. 293T cells are a human kidney epithelia cell line, which does not normally express WASp. Cells at 70% confluency were transiently

transfected with 20ug EGFP or EGFP-WASp using PEI as the transfecting agent harvested after 48 hours to allow protein expression, and the protein lysate separated on an acrylamide gel. When probed with an anti-WASp antibody a protein of ~90kDa was revealed (**Figure 3.3**) whereas a band of 65kD is normally seen for wt WASp in haematopoietic cells. The cells transfected with EGFP alone did not expose a band on the gel. The western confirms that the visible green fluorescent protein being expressed is recognised by an anti-WASp antibody and is the predicted size of a fusion of EGFP and WASp.

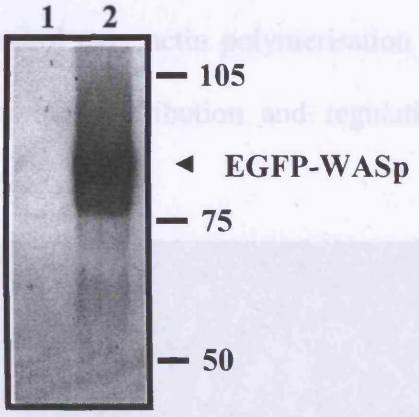


Figure 3.3 Western Blot of 293T cell lysates
Cell lysates prepared from EGFP (lane 1) and EGFP-WASp (lane 2) transfected 293T cells were separated by SDS-PAGE and probed for WASp using an anti-WASp antibody.

3.2.2 Transient transfection of human dendritic cells

WASp expression is restricted to cells of the haematopoietic lineage, so a readily available source of haematopoietic cells were transfected to see how the fusion protein would be expressed and what effects it would exert on the cell. Normal human dendritic cells derived from peripheral blood (a gift from S.Burns, ICH) were plated onto coverslips and transfected with EGFP or EGFP-WASp using LID (Hart et al., 1997) Fixed and permeabilised cells were stained for filamentous actin (F-actin) and nuclear staining. Overlaid colour images were captured on a CCD digital camera. Low-level

transfection was achieved with both constructs, which although was not quantified was clearly visible. Cells transfected with pEGFP-C2 expressed EGFP throughout the cell with distinct actin staining. Clearly visible in red were specialised actin structures, podosomes (**Figure 3.4a,b**). However when dendritic cells were transfected with EGFP-WASp there was no distinct EGFP or actin staining alone. There were however large aggregates within the cytoplasm that were yellow, showing co-localisation of actin and EGFP (**Figure 3.4c,d**). This shows that overexpression of the EGFP-WASp fusion protein is leading to dysregulation of actin in normal WASp containing haematopoietic cells, where previously control over actin polymerisation was tightly regulated. This ability to influence the normal distribution and regulation of F-actin within cells suggests the fusion is therefore active.

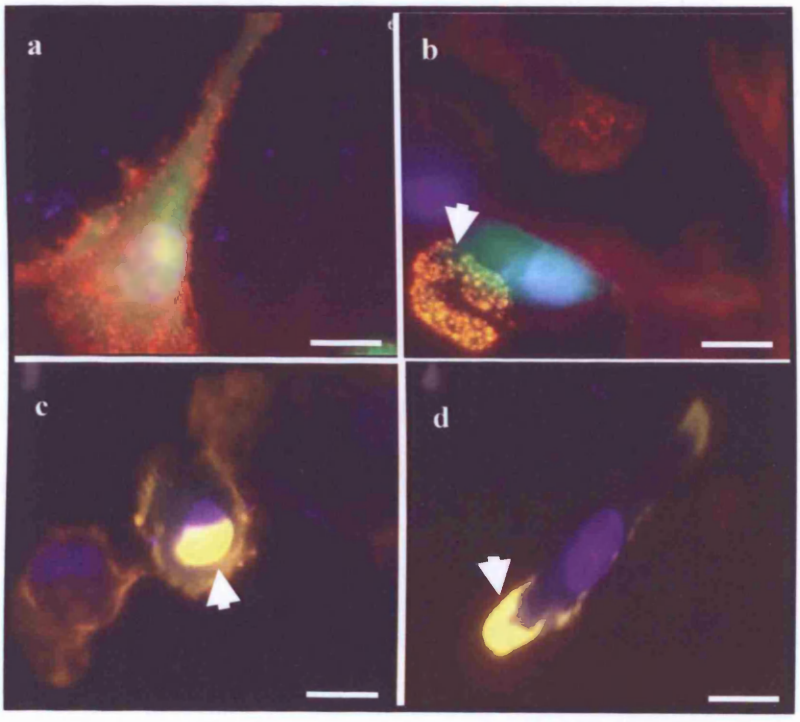


Figure 3.4 Transient transfection of human dendritic cells
Peripheral blood derived human dendritic cells were transiently transfected with EGFP (a+b) or EGFP-WASp (c+d) (green) using a lipid and integrin binding peptide complex. Cells were counterstained with rhodamine phalloidin (red) and DAPI (blue) to show F-actin and nuclei, respectively. Arrows point to podosomes in (b) and areas of co-localisation in (c) and (d). Overlaid images of all 3 colour channels were captured using a CCD digital camera. Scale bar is 20um

3.2.3 Generation of oncoretroviral cDNA constructs

The initial experiments proving expression of a fusion protein led us to further our approach towards a gene therapy protocol. Oncoretroviruses have the ability to integrate their genome into an infected cell, which is particularly useful to correct primary immunodeficiencies. A pluripotent stem cell can be permanently transduced which can divide to repopulate the immune system without the loss of transgene.

3.2.3.1 Subcloning EGFP-WASp into pBluescript

EGFP-WASp was removed from pEGFP-WASP-C2 with Nhe-I and BspMI, and blunted using the Klenow large fragment. This was then inserted into pBluescript (appendix 4), linearised with SmaI, a blunt cutter, and dephosphorylated to prevent re-closure. This had three benefits. Firstly it increased the number of restriction sites at either end of the EGFP-WASp expression cassette available for further subcloning, secondly as it was a blunt ligation, constructs in both orientations were obtained further increasing the restriction sites available for future use if needed. Thirdly, the poly A site was removed. This is necessary for full-length expression of the retrovirus mRNA needed for packaging and production of infective virus. This created two constructs designated pB-EW and pB-EWrev.

3.2.3.2 Cloning EGFP-WASP into a retrovirus backbone

The retroviral construct of choice was PINCO (Introna et al., 1998; Grignani et al., 1998a; Gasperi et al., 1999) (appendix 5) This retroviral construct was chosen for several reasons. It has a Molony Murine Leukaemia virus LTR and an internal CMV promoter, previously been shown to generate high levels of transgene expression, often an important requirement for gene therapy. In addition there is an EBV EBNA-1 ori

site. When the plasmid is transfected into producer cells this allows it to divide episomally with the cells without loss of plasmid, and therefore easy creation of a stable producer cell line without cloning of single cells. Thirdly, a puromycin resistance marker allows selection of transduced cells with puromycin in 3 days. Therefore, high titer viral producer cells to be made quickly and efficiently, again removing the need for cell cloning.

pB-EW was digested with HindIII and NotI and the fragments separated on a gel to remove EGFP-WASp. This fragment was sticky end ligated into PINCO-EGFP also digested with HindIII and NotI to replace the EGFP cassette (**Figure 3.5**) creating PINCO-EGFP-WASp (appendix 6). Cloning was confirmed by digestion with EcoRI, as extra recognition sequences are introduced, and separation on a 1% agarose gel (not shown).

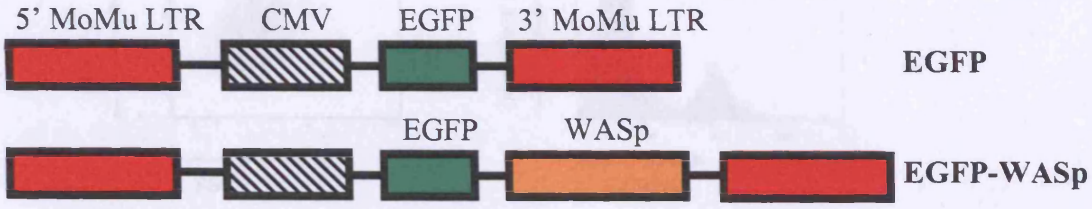


Figure 3.5. Cloning of retroviral PINCO vectors
EGFP-WASp was removed from pEGFP-WASp-C2 and blunt ligated into an intermediate pBluescript removing the poly A tail and increasing the potential number of cloning sites. EGFP-WASp was then ligated between the LTRs of the retroviral vector PINCO EGFP.

3.2.3.3 Production of high titer retrovirus

When Phoenix cells, a retrovirus producer cell line, are transfected with the PINCO retroviral vector construct a replication deficient oncoretrovirus is made. Ecotropic Phoenix cells, previously selected for 2 weeks in Diptheria toxin and hygromycin, to select for GAG and POL were transiently transfected by calcium phosphate precipitation with either PINCO-EGFP or PINCO EGFPWASp constructs and selected

with puromycin to give a high virus titer producing cell line. These cells were EGFP positive after 24 hours and the pattern of expression was identical to that previously seen in COS-7 cells (data not shown). After 5 days puromycin selection was halted and the viral supernatant harvested and titered on the permissive NIH 3T3 cell line. A representative flow cytometric plot showing the percent transduction at a dilution of 1:250 of PINCO EGFP-WASp is shown (**Figure 3.6**) from which a titer could be obtained. An infectious titer was calculated by multiplying the number of cells by the dilution, and dividing by the percentage positive for the transgene, in this case EGFP and EGFP-WASp. This gave a consistent titer of ~1x 10^6 for both EGFP and EGFP-WASp vectors.

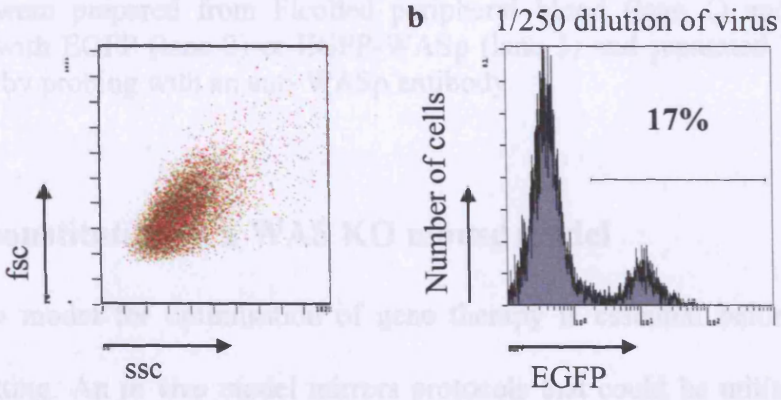


Figure 3.6 Flow cytometric plots of Infected 3T3 cells
50,000 3T3 cells were infected with increasing dilutions of retrovirus in a total volume of 1ml and harvested 72 hours later for flow cytometry. a) The forward (fsc) and side scatter (ssc) show the cell population. b) Percent EGFP expression was measured and used to calculate virus infectious titer.

When the pattern of EGFP-WASp expression was examined in the infected cells a similar cellular distribution of cytoplasmic staining was observed to transduced cells seen earlier (**Figure 3.7a**). An immunoblot of EGFP-WASp infected 3T3 cell lysate probed with an α-WASp antibody revealed a 90kDa band (**Figure 3.7b**), also observed previously, showing EGFP-WASp could be successfully packaged in a retrovirus and

subsequently expressed under the control of a viral LTR. The band in lane 3 at 60kDa is an artefact and appeared across 2 wells (**Figure 3.7b**).

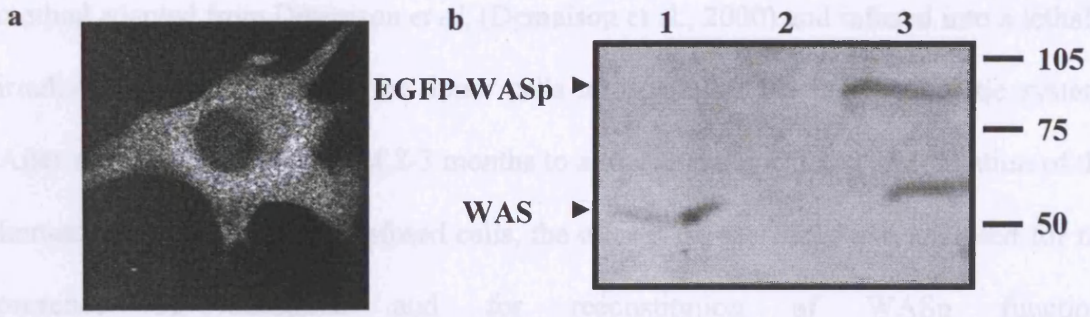


Figure 3.7 Confocal image and western blot of infected 3T3 cells
a) Infected cells were trypsinised and plated onto coverslips prior to fixation. Samples were mounted and z sections captured to reveal the pattern of EGFP staining. b) Lysates were prepared from Ficolled peripheral blood (lane 1) and 3T3 cultures infected with EGFP (lane 2) or EGFP-WASp (lane 3) and separated by SDS-PAGE followed by probing with an anti-WASp antibody.

3.2.4 Reconstitution of a WAS KO mouse model

An *in vivo* model for optimisation of gene therapy is essential before transfer to a clinical setting. An *in vivo* model mirrors protocols that could be utilised in a clinical setting, albeit in a murine environment, and successful recovery of a murine model is a pre-requisite before clinical trials can start. This allows optimisation of vectors, transduction protocols and efficiencies and meaningful analysis of successful reconstitution. Although our retroviral constructs contained human WASp fused to EGFP, it was thought that because of the high homology (~86%) between human and murine WASp (Derry et al., 1995) there would be cross-reactivity and human WASp would be able to reconstitute the mouse model.

The basic protocol used in the generation of an *in vivo* model is outlined in **Figure 3.8**. Briefly sca1+ murine stem cells from WASp KO mice were harvested from the bone marrow, infected *ex vivo* in the presence of cytokines on retronectin coated plates in a method adapted from Demaison *et al*, (Demaison et al., 2000) and infused into a lethally irradiated recipient to allow the donor cells to repopulate the haematopoietic system. After a period of incubation of 2-3 months to allow engraftment and repopulation of the immune system with the transfused cells, the mice were sacrificed and analysed for the presence of transgene and for reconstitution of WASp function.

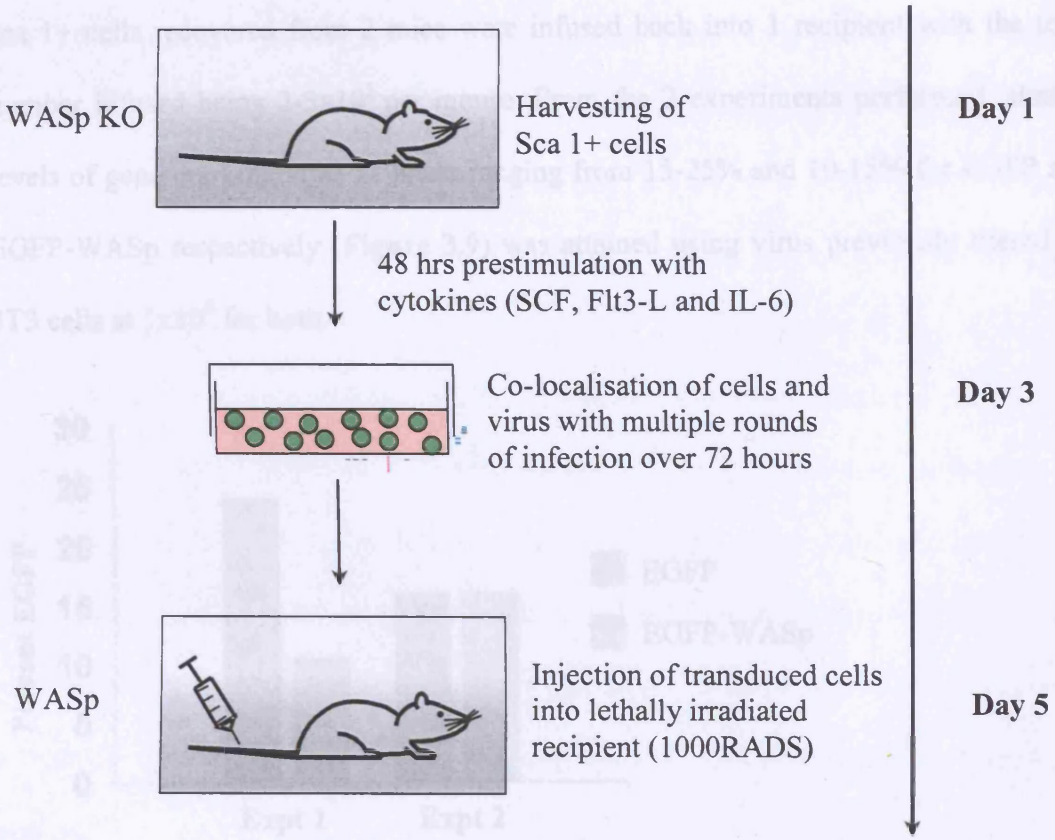


Figure 3.8 Schematic of transduction protocol for murine bone marrow transplant
Sca 1+ cells were harvested from the bone marrow of donor WASp KO mice and placed into culture for 48 hours in the presence of cytokines. The cells were then infected twice daily with retroviral supernatant in 24 well plates precoated with retronectin which had been pre- loaded with retrovirus. After 72 hours the cells were washed and injected intravenously into lethally irradiated WASp KO recipients. A sample of the cells was kept in culture for 72 hours to determine expression levels.

Sca 1 positive cells recovered from WAS KO mice were cultured in the presence of cytokines for 48 hours to induce mitosis before infection with ecotropic retrovirus. Virus was preloaded onto retronectin-coated plates and sca 1+ cells added before additional virus was added diluted 1:1 with fresh media and the relevant cytokines. The stem cells underwent a further 4 rounds of infection over a 48 hour period after which the cells were either infused into mice or kept in culture for a further 72 hours to asses the level of gene marking. As many cells as possible were re-introduced into lethally irradiated recipients to increase the chance of successful reconstitution. In general, the sca 1+ cells recovered from 2 mice were infused back into 1 recipient with the total number infused being $2\text{-}3x10^5$ per mouse. From the 2 experiments performed, similar levels of gene marking after 72 hours ranging from 15-25% and 10-15% for EGFP and EGFP-WASp respectively (**Figure 3.9**) was attained using virus previously titered on 3T3 cells at $1x10^6$ for both.

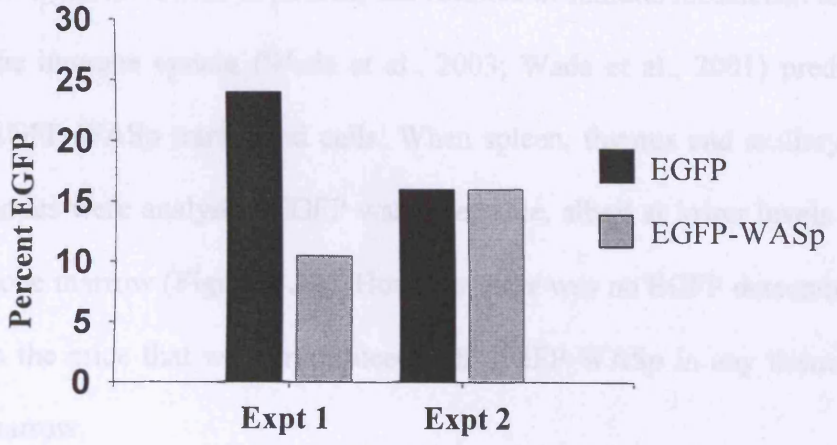


Figure 3.9 Transduction levels in Sca 1+ cells
Bar chart of EGFP transduction levels 72 hours after transplant of retrovirally infected Sca 1+ cells, determined by flow cytometry. Two experiments are shown where EGFP and EGFP-WASp containing vectors were used

3.2.4.1 Transduction efficiency after reconstitution

Reconstituted mice were sacrificed 2-3 months after infusion of transduced cells. Bone marrow, spleen, thymus and lymph nodes were removed and analysed for the presence of either EGFP or EGFP-WASp. The presence of transduced cells was first confirmed by flow cytometry of the bone marrow. Bone marrow from mice in experiment 1 revealed that mice reconstituted with EGFP still had 1-8% EGFP positive cells whereas those reconstituted with EGFP-WASp had no transduced cells detectable (**Figure 3.10**). Upon repetition of the experiment greater success was achieved with both EGFP and EGFP-WASp able to be detected in the bone marrow. The levels of EGFP were 7-33% and EGFP-WASp 0.4-5.9% (**Figure 3.10**). It is unclear why EGFP levels were retained close to the input levels whereas EGFP-WASp levels were significantly reduced compare to the input percent. It has been reported that there is a selective advantage in the homing capabilities of stem cells that express WASp (Lacout et al., 2003) and revertant mutations in patients has resulted in somatic mosaicism and partial recovery of the immune system (Wada et al., 2003; Wada et al., 2001) predicting an increase in EGFP-WASp transduced cells. When spleen, thymus and axiliary and brachial lymph nodes were analysed, EGFP was detectable, albeit at lower levels than was seen in the bone marrow (**Figure 3.11**). However there was no EGFP detectable by flow cytometry in the mice that were transduced with EGFP-WASp in any tissue other than the bone marrow.

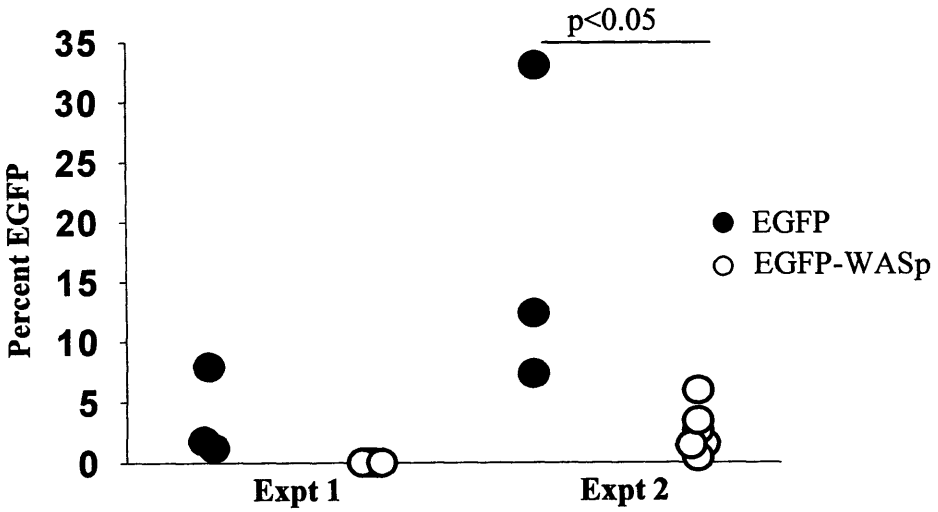
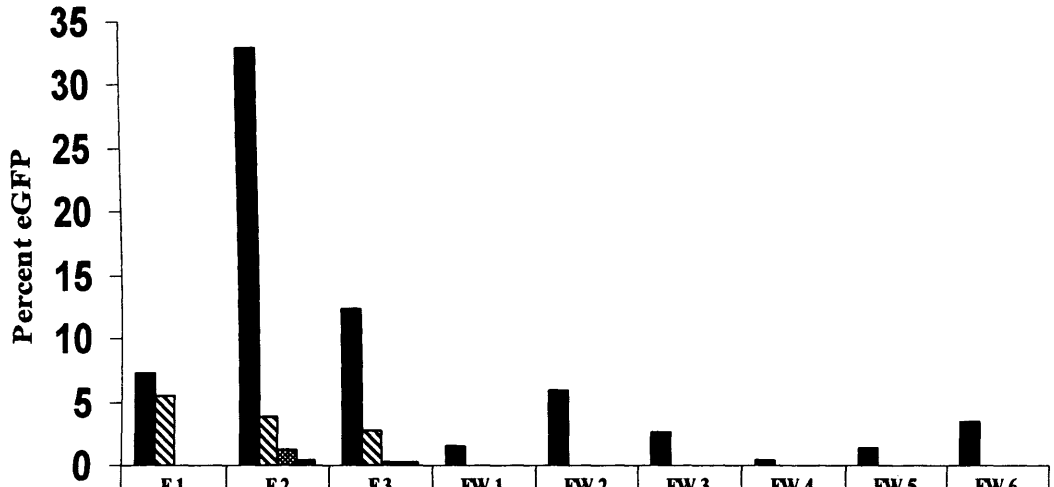


Figure 3.10 Transduction levels in reconstituted mice
Bone marrow was removed 2-3 months after mice were transplanted and the levels of transgene expression were assessed by EGFP flow cytometry. Two experiments are shown and each point represents an individual mouse. P value is from students T test



	E1	E2	E3	EW1	EW2	EW3	EW4	EW5	EW6
BM	7.3	33	12.3	1.5	5.9	2.6	0.4	1.4	3.4
Spl	5.45	3.8	2.8	0	0	0	0	0	0
Thy	0	1.2	0.25	0	0	0	0	0	0
LN	0	0.45	0.3	0	0	0	0	0	0

Figure 3.11 Transgene expression in secondary lymphoid tissue
2-3 months post transplant mice were sacrificed and the levels of transgene expression determined by flow cytometry detecting EGFP levels in bone marrow (BM), spleen (Spl), thymus (Thy) and lymph node (LN) single cell suspensions. E = EGFP and EW = EGFP-WASp. Numbers shown are the percentages represented in the bar chart.

3.2.4.2 Analysis of phenotypic recovery

To assess the quality and quantity of recovery of phenotype, bone marrow from the reconstituted mice was put into culture with GM-CSF followed at day 3 by addition of IL-4 which, by day 7, would generate dendritic cells (de Noronha et al., 2005). These cells were then positively sorted for transduced cells by FACS on the basis of EGFP level. After sorting the positive cell population was increased from 5% to >95% (**Figure 3.12**). Sorted cells were plated onto coverslips, allowed to adhere, then fixed and stained with phalloidin to assess the F-actin distribution and podosome levels in the cells. Murine dendritic cells from WAS KO mice are largely deficient in clearly defined podosomes (~5%) compared to 50-60% we have observed in Sv/129 normal control mice.

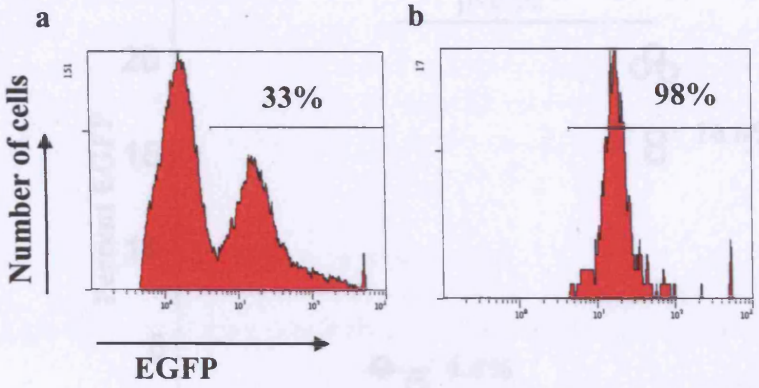


Figure 3.12 Fluorescence Activated Cell Sorting of murine bone marrow
Representative FACS plots showing purification of EGFP positive cells from murine bone marrow derived dendritic cells from transplanted mice 2-3 months post transplant. Cells pre-sort (a) were purified to >95% purity (b) post sort, based on EGFP levels.

Restoration of podosomes was therefore a functional test of recovery of phenotype in addition to EGFP-Wasp expression. Confocal images of dendritic cells made from mice transduced with EGFP revealed high levels of EGFP but podosomes were found in only 4.4% of all cells counted (**Figure 3.13**). In EGFP-WASp expressing dendritic cells the

percent of cells with podosomes ranged from 8.1-20.5% with an average of 16.6% (**Figure 3.13**). An increased level of transduced cells present in the bone marrow did not necessarily correlate with an increase in the percent of podosomes (e.g. mouse 4 had 0.4 % transduced cells, but 19.6% of those had podosomes). Reconstitution of podosomes observed was up to a third of that seen in normal Sv/129 controls, but may be sufficient for a beneficial therapeutic effect. In addition to having podosomes there was co-localisation between EGFP and the F-actin core of the podosomes showing that the introduced EGFP-WASp, as has been seen in human macrophages (Jones et al., 2002) and dendritic cells (Burns et al., 2001a), was integral to *de novo* podosome formation (**Figure 3.14**).

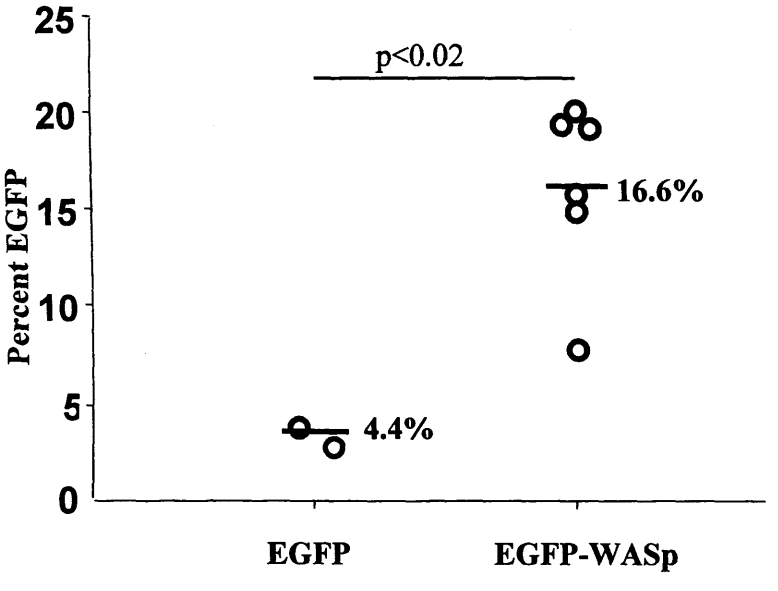


Figure 3.13 Percent of murine bone marrow derived dendritic cells with podosomes
Bone marrow derived dendritic cells from experiment 2 were positively sorted for EGFP and plated onto coverslips for 24 hours to allow cells to adhere. Cells were then fixed and counterstained with rhodamine phalloidin to visualise F-actin. Confocal images of the cells were obtained and maximal projections of 6-12 z sections were used to score for the presence or absence of podosomes on the basal surface. Each point represents an individual mouse with 50-100 cells counted for each. P value is from a students T test.

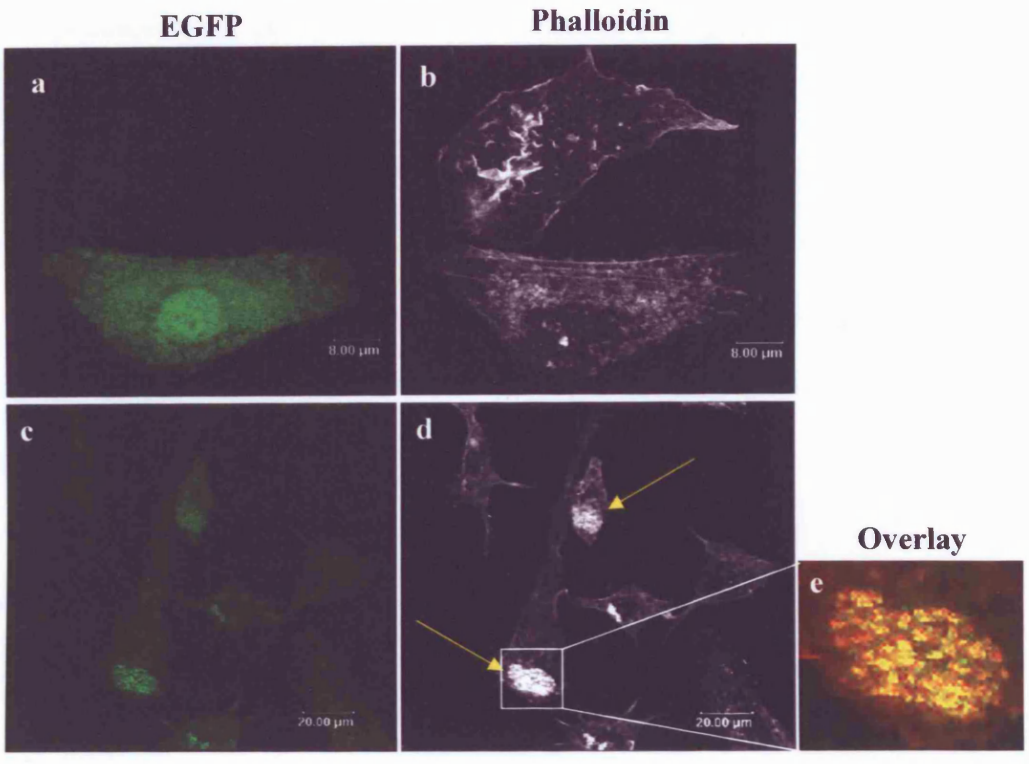


Figure 3.14 Confocal images of bone marrow dendritic cells
Bone marrow dendritic cells from EGFP (a + b) and EGFP-WASp (c + d) transduced mice were positively sorted on the basis of EGFP before being plated onto coverslips overnight. Cells were fixed and counterstained with rhodamine phalloidin to visualise F actin. Images are maximal projections of 6-12 z sections. An overlaid image of EGFP (green) and phalliodin (red) (e) shows co-localisation in yellow.

3.2.4.3 Quality of WASp reconstitution

Although successful transduction and subsequent reconstitution in the bone marrow could be observed in 6/6 mice in experiment 2, the quality of this reconstitution needed to be assessed. Although few WASp null cells have podosomes, it has been shown recently that in XLT, a mild form of WASp where there is reduced WASp expression levels, there are podosomes present but in reduced numbers per cell compared to normal (Linder et al., 2003). Therefore the number of podosomes per cell in dendritic cells from transduced mice could relate to the amount of WASp expressed and subsequently the quality of reconstitution compared to normal and whether the levels we achieve are sufficient to correct the phenotype.

The number of podosomes per cell for normal dendritic cells averaged 30.4 compared to 15 for WAS KO, when only the cells with podosomes are included (**Figure 3.15**). Dendritic cells from EGFP transduced cells had an average of 12 podosomes per cell again when only scoring those containing podosomes. When dendritic cells from EGFP-WASp reconstituted mice were compared, the average number of podosomes per cell ranged from 14-40 for the 6 mice, with 5/6 mice showing the quality of reconstitution to be at normal levels. When the podosomes per cell data from all 6 EGFP-WASp transduced mice was pooled to increase the numbers the average number per cell was 30.9 (**Figure 3.15**).

The number of podosomes per cell was divided into groups of 0, 1-20, 21-50, 51-100 and >100 and the percent of cells in each increment plotted as previously shown in (Linder et al., 2003) to analyse the natural range and distribution of podosome number in the whole population. Due to the low numbers of cells counted for each mouse all 6 EGFP-WASp transduced mice were again pooled to give an average.

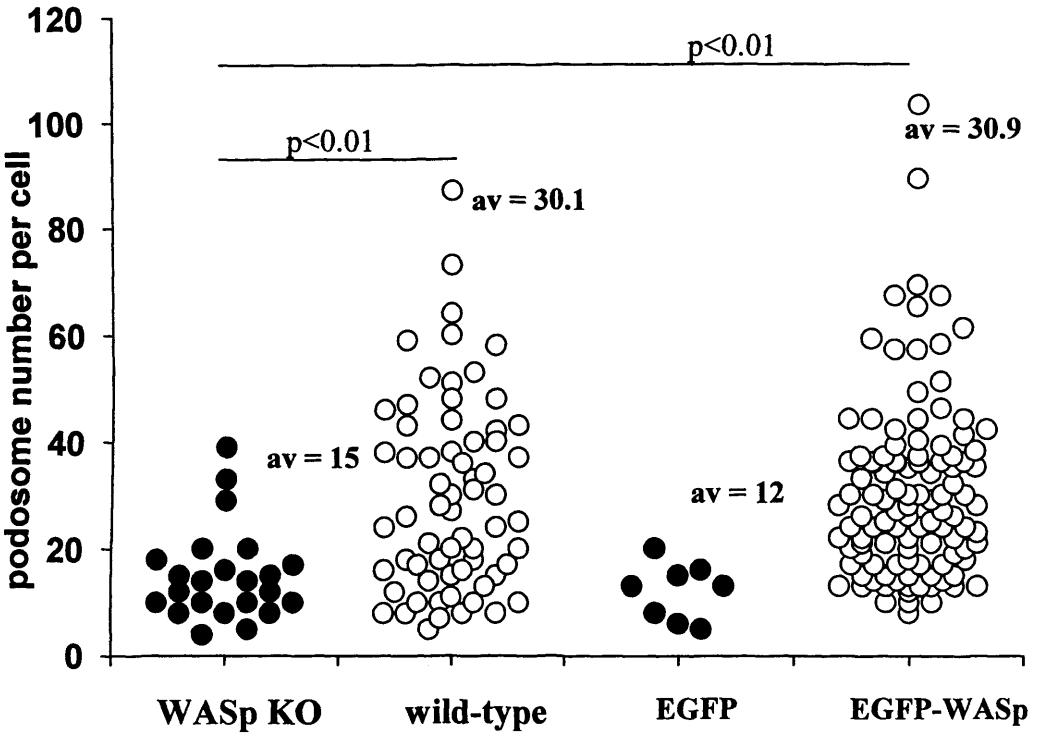


Figure 3.15 Podosomes per cell in dendritic cells
Cells were stained with phalloidin to visualise actin and therefore podosomes. Positive cells had the number of podosomes per cell scored and plotted. Each point represents a single cell with podosomes present. Where necessary values are pooled from similar treated mice (EGFP n=2, EGFP-WASp n=6). P values shown are from a students T test

When plotted against normal and EGFP transduced mice the range of podosomes per cell was very similar to normal (**Figure 3.16**) again suggesting the quality of reconstitution was approaching normal.

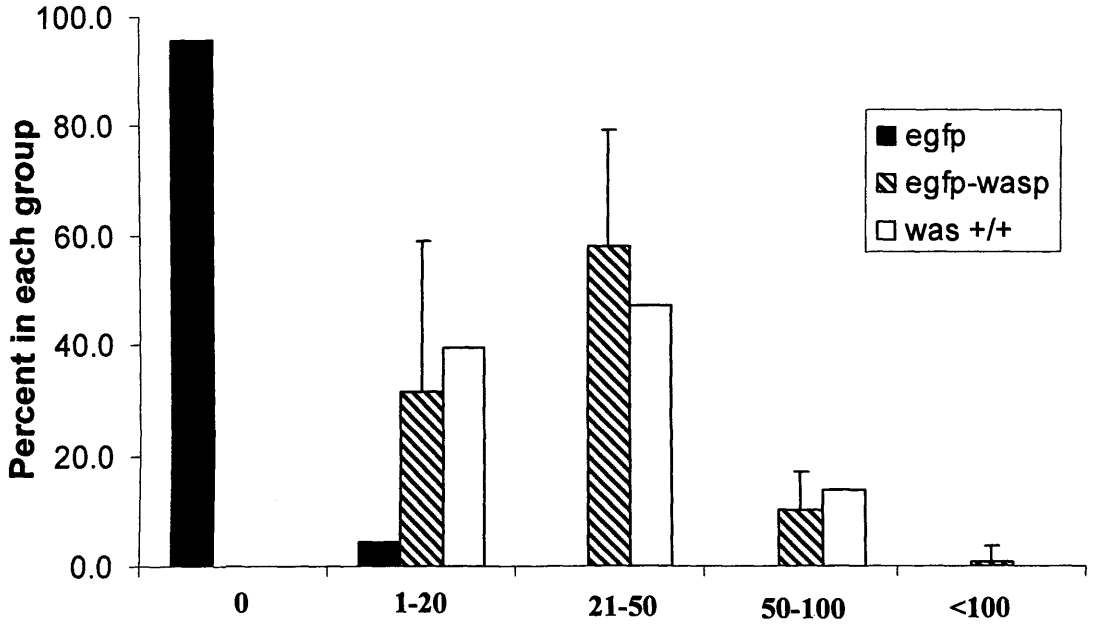


Figure 3.16 Distribution of podosome number in a cell population
Bar chart representing the distribution of podosomes in EGFP+ sorted murine bone marrow dendritic cells from transplanted mice compared to normal Sv/129 mice. EGFP-WASp and normal values are for cells positive for podosomes only. Bar chart values are from 50-150 cells and are the combined values from similar treated mice. EGFP (n=2) EGFP-WASp (n=6). Error bars shown are +1 SD

3.3 Discussion

The aim of this work was to firstly make a fusion protein of EGFP and human WASp that would enable direct visualisation of WASp expression in live cells, providing a useful tool for molecular investigations into the activity of WASp. Secondly it would be of use in optimising a gene therapy protocol, as EGFP is readily detectable using fluorescence microscopy and flow cytometry. Although WASp has been FLAG tagged previously at the C-terminus (Symons et al., 1996), the important protein inhibitory folding structure meant an N-terminal EGFP fusion was needed. A fusion of EGFP and WASp was cloned and when transfected into cell lines expressed a protein, which was detected with anti-WASp antibodies at a size of 90kDa compared to the usual 65kDa

seen for wild-type WASp. The expression pattern of WASp has been described as predominately cytosolic (81%) with some membrane bound (16%) and very little in the nucleus (3%) (Rivero-Lezcano et al., 1995). The pattern of fluorescence seen in adherent cells transfected with EGFP-WASp mimicked this pattern of expression suggesting a functional fusion was being expressed and that the EGFP was having little or no effect upon the function of WASp.

WASp is haematopoietic restricted (Derry et al., 1994) so EGFP-WASp was transfected into normal human dendritic cells, a haematopoietic cell available at the time to observe any effects. This had a major effect on the actin structure of transfected cells. Normal dendritic cells express specialised actin structures, podosomes, whose assembly are dependant upon the presence of WASp (Linder et al., 1999; Burns et al., 2001a). When EGFP-WASp was transfected into dendritic cells there was a loss of podosomes and an aggregation of the actin. Changes in the regulation of WASp can dramatically alter the appearance and the number of podosomes in dendritic cells. Use of the constitutively active V12Cdc42 leads to disruption of podosomes in human macrophages, as does overexpression of the VCA domain (Linder et al., 2000). Thus overexpression of an EGFP-WASp fusion protein may be acting in a similar manner, showing the fusion is active.

EGFP-WASp was cloned into a retroviral vector for use in *ex vivo* gene therapy protocols and infectious titers of $1x10^6$ regularly obtained. The pattern of expression of EGFP-WASp in retrovirally-infected cells was similar to that observed in transfected cells and immunoblots revealed a 90kDa band when probed for WASp. This shows viral

vectors encoding for EGFP-WASp are capable of infecting cells and the cells subsequently capable of expressing a functional fusion protein.

When PINCO EGFP-WASp cDNA was microinjected into normal human dendritic cells it co-localised to the site of podosomes. Furthermore when human WASp null dendritic cells were microinjected with EGFP-WASp they made podosomes where previously they did not (Burns et al., 2001b). Again there was co-localisation of EGFP and actin in these newly formed podosomes. This confirms other groups' work (Linder et al., 1999) that WASp is required for podosome assembly and disassembly and the EGFP-WASp fusion is active.

Retrovirus infection and integration of the transgene is limited because of the need for the breakdown of the nuclear membrane, which occurs when cells undergo mitosis (Miller et al., 1990b; Roe et al., 1993). Although expression was confirmed in fibroblast cell lines, WASp is normally haematopoietic restricted. This limits the use of a retrovirus *in vitro* due to the lack of an available WASp null cell line in which to show reconstitution and restoration of function when WASp is introduced. There has been some limited success *in vitro* where WASp null BLCL's have been transduced with retrovirus particles containing WASp and had restoration of microfilopodia on the cell surface (Candotti et al., 1999; Wada et al., 2002) and in T cells where CD3 mediated proliferation has been restored (Wada et al., 2002).

Because of a lack of available haematopoietic WAS KO cells to use *in vitro* an *ex vivo* gene therapy protocol was devised where transduced murine stem cells from WAS KO mice were introduced into lethally irradiated WAS KO recipients and allowed to

repopulate the haematopoietic system, with the intention of correcting the defects seen in WAS KO mice. Murine sca1+ stem cells could be transduced at levels up to 25% measured by EGFP expression, which is comparable to levels found by other groups (Strom et al., 2003b; Klein et al., 2003), which has lead to recovery of a normal phenotype.

After 3 months there was significant gene marking with EGFP in 6/6 mice. There was also expression of EGFP-WASp in the bone marrow in 6/9 mice although it was lower in EGFP-WASp transduced mice. There was higher initial gene marking in the second experiment that led to a higher level after 3 months in mice when compared to the lower transduction efficiency in the first experiment. This would suggest the higher the initial transduction the better the reconstitution with transduced cells. It is unclear why there are lower expression levels in EGFP-WASp transduced cells compared to EGFP when the starting populations had similar levels of transduction. It has been previously shown that there is a selective advantage for homing in cells that express WASp compared to WASp null cells and somatic mosaicism has revealed a growth advantage for T cells. The results here do not confirm or dispute any growth advantage.

As expression of EGFP-WASp in normal dendritic cells leads to aggregation of actin and loss of normal cytoskeletal architecture, a similar effect may be exerted on WASp KO cells with high expression levels. The combined effect of a viral LTR and the internal CMV leads to high expression *in vitro* and these promoters may lead to too much WASp expression leading to abnormal actin regulation and the death of cells. In fact the EGFP-WASp mean fluorescence intensity (MFI) was less than EGFP (MFI) in transduced bone marrow and confocal images of dendritic cells from transduced mice

were generally less bright, suggesting only cells that express EGFP-WASp at a relatively low level have survived, whereas high level EGFP expressing cells are viable.

Analysis of the transduction levels in secondary lymphoid organs showed that there was a significant decrease in the levels of transduced cells for EGFP and undetectable for EGFP-WASp. This seems to be linked to levels in the bone marrow as mice with higher levels here have increased levels in other organs. It is unknown why the levels in other lymphoid organs are reduced. There may not have been enough time to repopulate or there could be physical barriers preventing repopulation. Transduction levels similar or lower may be expected for EGFP transduced cells where there is no selective advantage compared to untransduced WAS KO cells, however if there is a selective advantage in cells that have WASp as has been previously shown from somatic mosaicism (Wada et al., 2001; Wada et al., 2003; Wada et al., 2004) it is not yet materialising in the reconstitution of the bone marrow or secondary lymphoid tissues. In addition it has been shown that the proposed selective advantage is cell type dependant with the presence of WASp more advantageous to T cell survival than B cell (Konno et al., 2004).

Bone marrow derived dendritic cells from EGFP-WASp transduced mice had podosomes restored in their cytoplasm and co-localisation of actin and EGFP-WASp showed the introduced WASp was crucial in this. Although the selected cells were <95% positive for EGFP-WASp there was only a third of the restoration of cells with podosomes compared to normal and there was no correlation with percent of cells transduced and podosome number. This may be sufficient for beneficial recovery of some actin regulation as low level WASp expression compared to no expression results in the less severe XLT phenotype (Linder et al., 2003).

Encouragingly when the quality of the reconstitution was measured by counting podosome numbers within the cells it was found to be of similar level to that seen in normal controls even though the levels of transduction were low. Furthermore the distribution of podosome number in cells was similar to that seen in normal mice showing there was no skewing of results due to a small population of highly transduced cells. This suggests there is a normal regulation of WASp in the cells and EGFP is not affecting the binding partners of WASp.

Using a retroviral vector we have shown that it is possible to reconstitute a WAS KO mouse model with EGFP-WASp and gain restoration of podosomes, an actin structure dependant on the presence of WASp, to normal numbers within dendritic cells. This was achieved with relatively low levels of transduction, upon analysis after 3 months.

If a greater percentage of stem cells could be transduced initially then there is the potential for higher levels of reconstitution which could lead to detection of transduced cells in spleen, thymus and lymph nodes thus potentially correcting all the defects seen in WAS.

There are two main problems associated with the use of retroviral vectors. The first is retroviral integration only occurring in dividing cells (Roe et al., 1993; Miller et al., 1990b), coupled with the relatively short half-life of the virus (Andreadis et al., 1997). There is a limited opportunity to infect true multipotent progenitor cells, as they have to be stimulated into cycle. The second problem relates to the favoured sites of integration of retroviruses. It has been shown that retroviruses prefer to integrate at gene promotor sites (Laufs et al., 2003; Wu et al., 2003) thus leading to the potential for oncogenic

events due to gene over or under expression. Such serious adverse events have occurred in a clinical trial in France where SCID-X1 trials using retroviral vectors (Hacein-Bey-Abina et al., 2003b). In that study 3 of 11 patients developed T cell leukaemia. In the first 2 patients, the T cell clones were shown to have retroviral insertions in the LMO-2 proto-oncogene promoter which lead to abnormal LMO-2 protein expression (Hacein-Bey-Abina et al., 2003b), the third patient is still being analysed. The abnormal LMO-2 expression was thought to be a direct result of the LTR enhancer activity in close proximity to the LMO-2 promoter. The LMO-2 integrant clones are thought to be selected via a growth advantage. The leukaemia may be partly due to the capacity of the T precursors to expand to fill the empty compartment and the age of the recipients as they were the youngest of the patients treated. This means improved strategies may be required. The use of retroviral vectors with improvements in vector design relating to self-inactivation and promoters and use of post transcription regulatory elements could lead to improved transduction efficiencies and higher levels of correction. It was decided however, although success was obtained with oncoretroviral vectors, to use lentiviral vectors to try and optimise and possibly improve a gene therapy protocol for WASp. This has the benefit of allowing *in vitro* experiments on WAS KO primary non-dividing cells to improve vector design and transduction protocols prior to proceeding to an *in vivo* setting.

CHAPTER 4

IN VITRO DEVELOPMENT OF LENTIVIRAL VECTORS

4.1 Introduction

As lentiviral vectors do not require the breakdown of the nuclear envelope to integrate its genome (Naldini et al., 1996) we have used self-inactivating lentiviral vectors containing strong viral promoters or putative endogenous *WAS* promoter sequences to transduce murine *WAS* KO dendritic cells and human WASp null patient macrophages *in vitro* with EGFP, EGFP-WASp and WASp in order to determine which is the most suitable for use in a murine stem cell reconstitution model (described in Chapter 3) and subsequent possible clinical trials.

Additional modifications to enhance transgene expression have been included in the lentiviral vector. A central poly-purine tract (cPPT) has been added to increase the stability of the mRNA and therefore increase titer. It has been shown that introduction of the cPPT element leads to higher transgene expression (Demaison et al., 2002). In addition some vectors have the woodchuck post-transcriptional regulatory element (WPRE) added 3' of the transgene, again thought to increase the stability of the mRNA (Zufferey et al., 1999). For additional safety this has been mutated to remove the start codon of the X-protein encoded by this sequence, which could be linked to oncogenesis (Kingsman et al., 2005). The WPRE has also been shown to increase the expression of the transgene in transduced cells (Zufferey et al., 1999; Demaison et al., 2002).

It has been shown that two sequences 5' of the WAS start codon, one of 500bp and a larger one of 1600bp, have been shown to have some promoter activity (Hagemann and Kwan, 1999; Petrella et al., 1998) in haematopoietic cell lines and is theorised to lead to a more physiologically regulated level of WASp expression compared to strong viral

promoters. Regulation of WASp expression could be important as high cellular levels of EGFP-WASp so far has lead to dysregulation of the actin cytoskeleton (Chapter3). The haematopoietic restriction of WASp (Martin et al., 2005) could also be in part due to this promoter sequence and could be useful for future work and other applications where lineage restriction may be important.

4.2 Results

4.2.1 Cloning lentiviral vectors containing viral promotors

Lentiviral vectors containing EGFP as the transgene were obtained (from K. Parsley Molecular Imunology Unit ICH) under the transcriptional control of either a CMV promotor or the 3' LTR from Spleen Focus Forming Virus. Briefly, because of a lack of suitable restriction sites in the lentivirus vectors and in EGFP-WASp-C2 to allow further cloning, EGFP-WASp was subcloned into a bluescript vector containing a SFFV LTR-EGFP-WPRE cassette (pB-SEW)(a gift from K.Parsley, Molecular Immunology Unit, ICH), originally made to clone SEW (**Figure 4.1b**). EGFP-WASp was excised from pB-EWrev an intermediate vector made when cloning PINCO EGFP-WASp (see chapter 3) via BamHI and HindIII and sticky end ligated into pB-SEW also digested with BamHI and HindIII to replace EGFP with EGFP-WASp to create pB-SEWW. Then, the EGFP-WASp-WPRE cassette from pB-SEWW was excised via BamHI and KpnI and sticky end ligated into the lentiviral vectors pHR'sincpptCEW (**Figure 4.1a**) (appendix 7) or pHR'sincpptSEW (**Figure 4.1b**) (appendix 8), also digested with BamHI and KpnI to replace EGFP-WPRE. The lentiviral vectors created were designated CEWW and SEWW depending on the promoter (**Figure 4.1a**, **Figure 4.1b** and appendix 9 and 10). HindIII digestion and separation on an agarose gel confirmed the cloning (not shown).

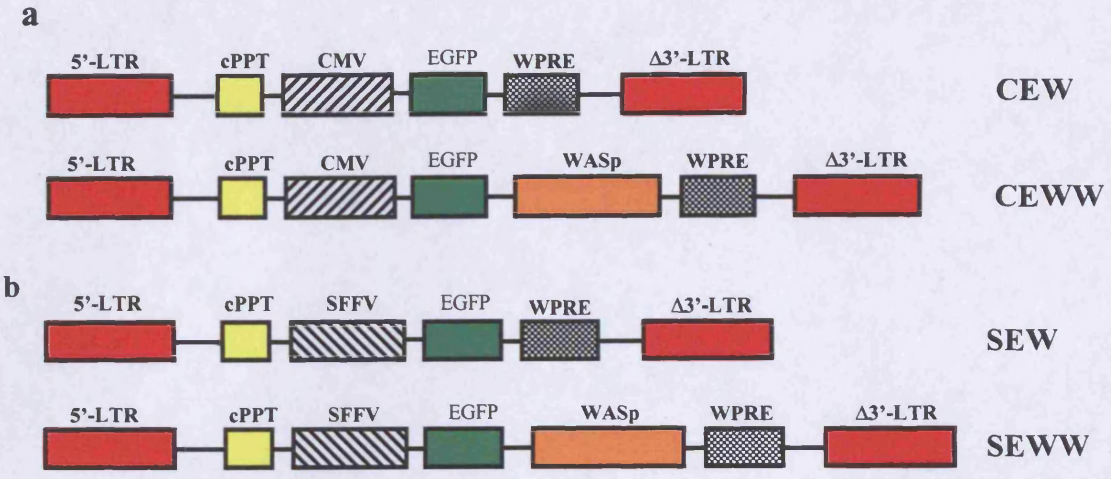


Figure 4.1 Lentiviral vectors containing CMV and SFFV internal promoters
Cartoon of intermediate pBluescript subcloning vectors containing a) EGFP and EGFPWASp. Cartoon depiction of lentiviral transfer vectors containing a) CMV promoter or b) SFFV as the internal promoter.
Also shown are the major features. EGFP-WASp-WPRE was cloned from the intermediate pBluescript vector using BamHI and KpnI. LTR = Long Terminal Repeat, cPPT = central Poly Purine Tract, WPRE = Woodchuck Post Transcriptional Regulatory Element.

4.2.1.1 Transduction of 3T3 cells with Lentivirus

Although during transient transfection during virus production, the producer cells expressed EGFP, in order to confirm there was infectious virus, 3T3 cells were infected. Confocal microscopy showed expression of EGFP and EGFP-WASp (**Figure 4.2a**) with a pattern of expression similar to that seen previously with retrovirally transduced cells (Chapter 3). Furthermore there was substantial disruption of the actin cytoskeleton with large aggregates of actin and EGFP-WASp not seen with EGFP (**Figure 4.2a**) also previously seen and has been reported with over expression of WASp in other cell lines (Symons et al., 1996). When an immunoblot of the cell lysate from transduced cells was probed with an anti WASp antibody there was a large protein revealed at 95kD compared to a band at 65kD seen for wild-type WASp in peripheral blood (**Figure 4.2b**).

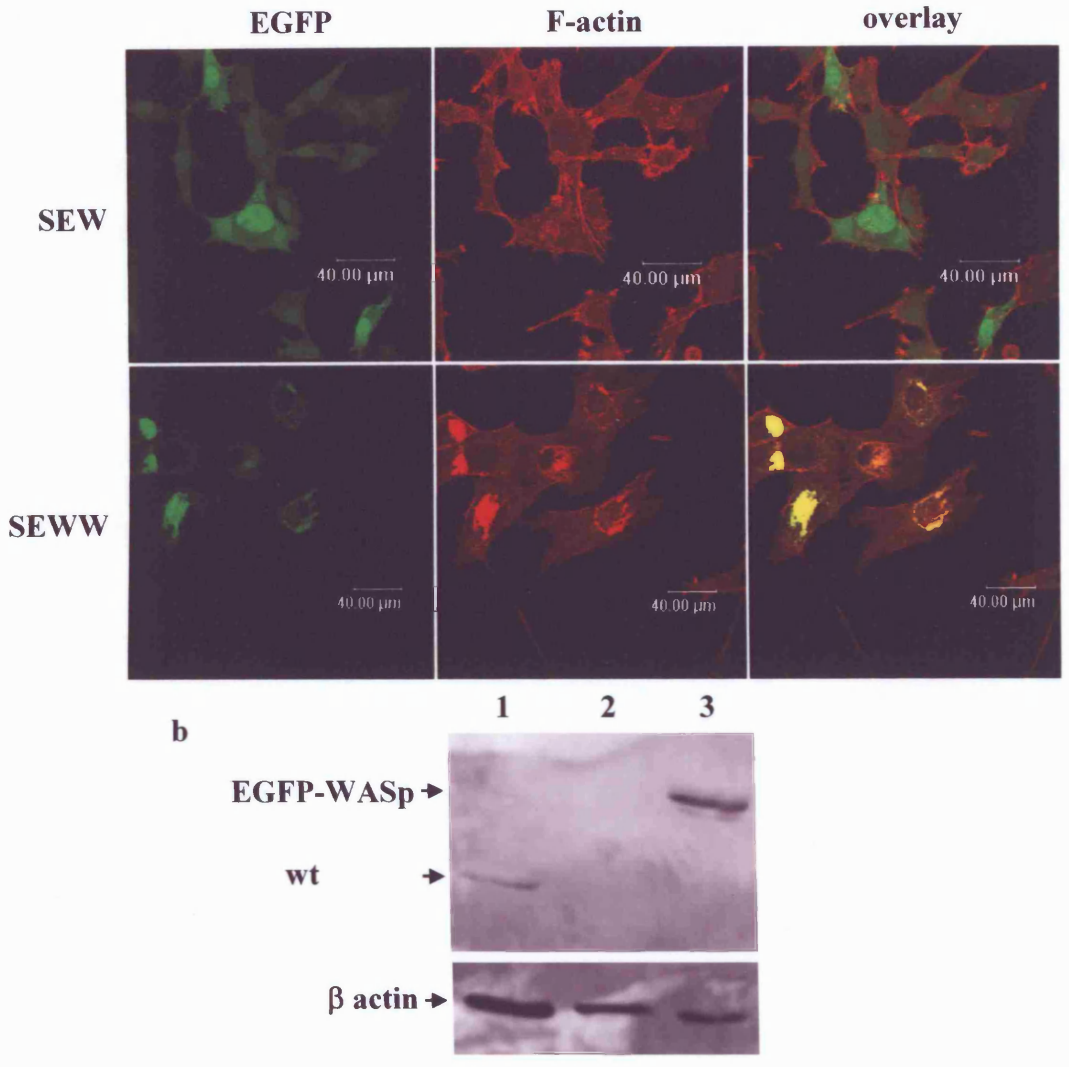


Figure 4.2 Infection of 3T3 cells
3T3 cells seeded onto coverslips were infected with lentiviral constructs containing EGFP (SEW) or EGFP-WASp at an MOI of 10. After 72 hours the cells were fixed and stained for F-actin. a) Images shown are representative confocal maximal projections of 8-12 Z sections. Green = EGFP, red = rhodamine phalloidin. b) Immunoblot of 3T3 cell lysate probed with anti WASp antibody showing EGFP-WASp expression. Lane 1 PBL, lane 2 SEW, lane 3 SEWW

4.2.1.2 Restoration of podosomes using lentiviral vectors *in vitro*

The lentiviral vectors were tested *in vitro* on WASp KO primary dendritic cells. Reconstitution of podosomes was used as a measurement of vector efficacy. Firstly a comparison of CMV and SFFV promoter driven vectors was performed to provide data based upon high expression levels.

Bone marrow derived dendritic cells were plated onto fibronectin coated glass coverslips and infected with CEW or SEW at an MOI of 100. Transfection levels were measured by flow cytometry, or by eye under a fluorescence microscope, and were consistently up to 50% positive for EGFP for both CMV (**Figure 4.3**) and SFFV (not shown) containing vectors

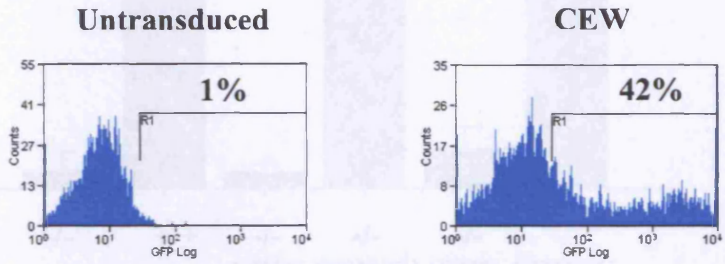


Figure 4.3 Infection of DC's with lentiviral vectors
Murine dendritic cells were infected on coverslips at an MOI of 100 and incubated for 72 hours to allow transgene expression. Cells were harvested and transduction efficiency determined by flow cytometry. CEW = CMV EGFP WPRE, SEW = SFFV EGFP WPRE

Normal murine dendritic cells *in vitro* make podosomes on the ventral surface in 50-70% of cells, whereas WASp KO dendritic cells have severely reduced numbers (>5%) (**Figure 4.4**). When WASp KO dendritic cells are transduced with EGFP there is no increase in the number of cells with podosomes whereas transduction with EGFP-WASp from vectors containing either CMV or SFFV showed a marked increase in podosome levels to 36% and 25%, respectively (**Figure 4.4**).

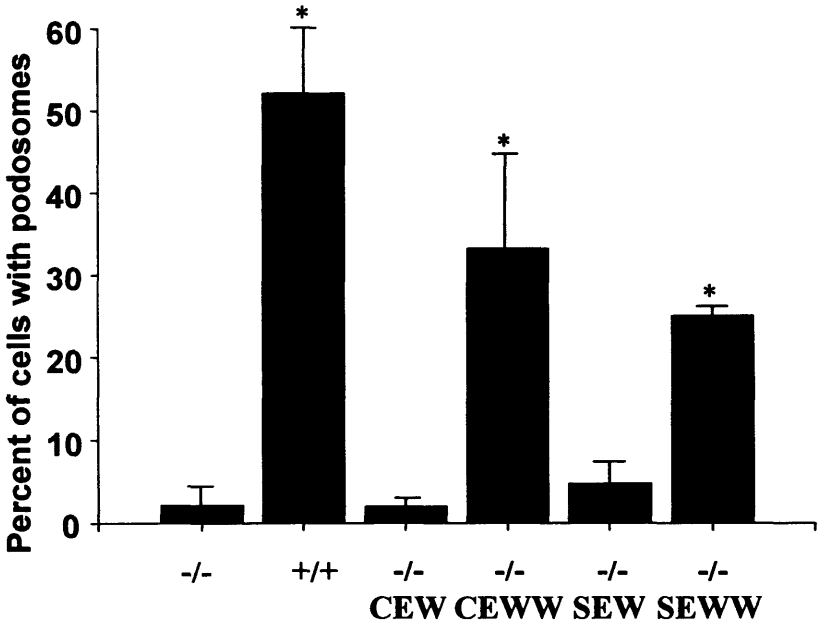


Figure 4.4 Reconstitution of podosomes
Bone marrow derived dendritic cells were seeded onto glass coverslips and infected at an MOI of 100 with EGFP (CEW, SEW) or EGFP-WASp (CEWW, SEWW). The cells were then fixed and permeabilised before staining with rhodamine phalloidin to visualise F actin. Confocal images of the cells were scored for the presence or absence of podosomes on the basal surface. 100-200 cells were scored. Error bars represent + 1 SD. * $p<0.001$ compared to value obtained for WAS KO cells.

Normal dendritic cells make podosomes that have a characteristic ring of vinculin around an actin core, whereas WASp KO dendritic cells do not. Although podosomes could be identified by the actin staining, the vinculin staining was often weak and was often only visible as a co-localising stain (**Figure 4.5a**) Infection with vectors expressing EGFP does promote podosome formation (**Figure 4.5b**). In EGFP-WASp transduced cells the fluorescence was reduced and was predominantly co-localised with F-actin at the sites where podosomes were located, often at the leading edge of the cell. There was vinculin staining at the podosome sites as seen in normal dendritic cells, suggesting normal assembly within the cell (**Figure 4.5c,d**)). The assembly of podosomes when EGFP-WASp is introduced has also been observed in human WASp null dendritic cells where DNA was microinjected (Burns et al., 2001b) supporting the findings here.

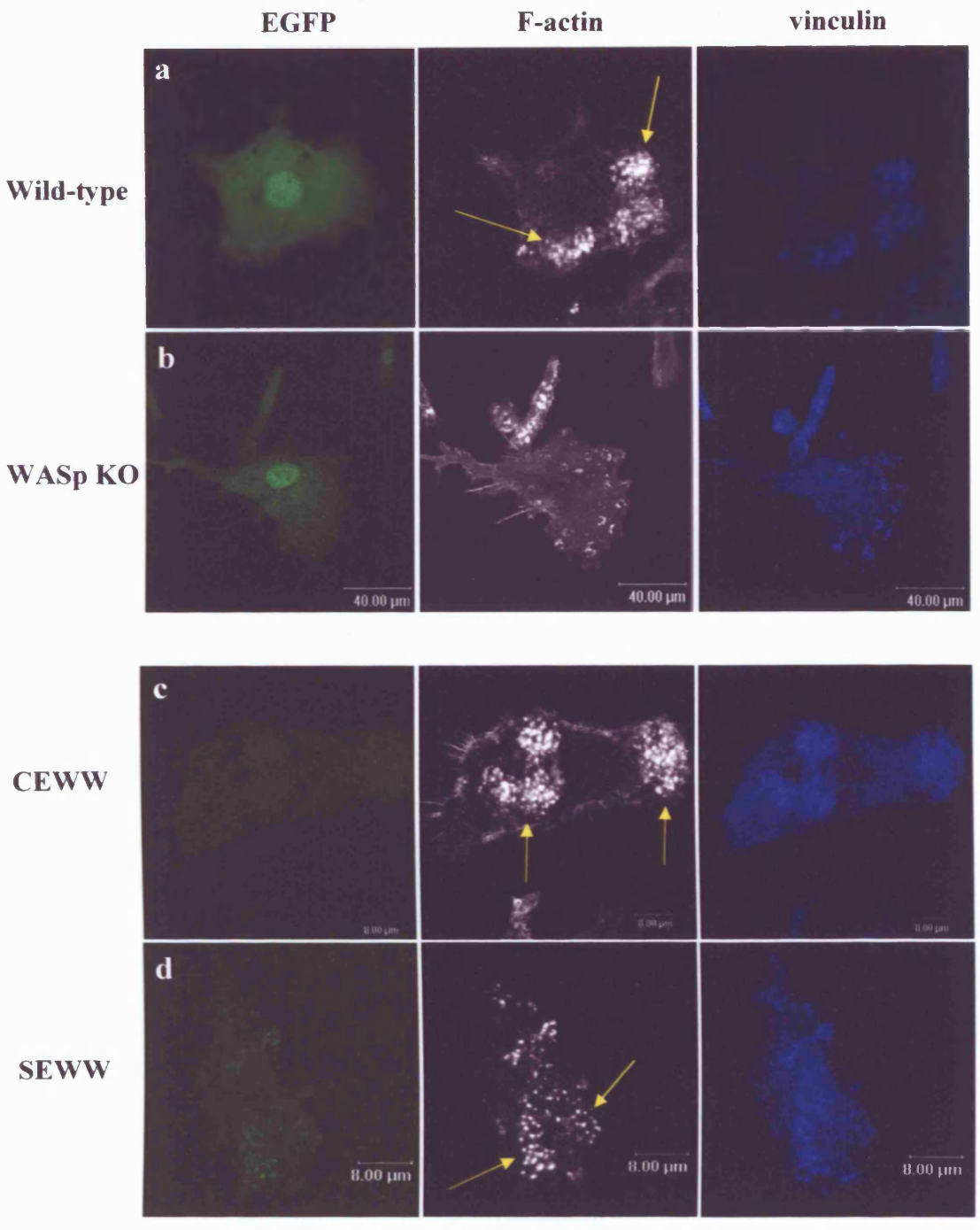


Figure 4.5 Confocal images of EGFP transduced murine dendritic cells
Bone marrow derived dendritic cells from WASp wild-type or WASp KO mice were plated onto glass coverslips and infected at an MOI of 100 with (a+b) SEW, (c) CEWW or (d) SEWW. After 72 hours the cells were fixed in PFA and permeabilised before staining for F-actin and vinculin. Cells represented are the maximal projections from 8-12 Z sections showing EGFP (green), F-actin (white) and vinculin (blue). Podosomes are visible in control and EGFP-WASp transduced cells (yellow arrows) but not WASp KO cells.

4.2.1.3 Quality of reconstitution

To assess how well the expression of WASp from either promoter corrected the phenotype of WASp KO dendritic cells, the number of podosomes present in each transduced cell with podosomes was counted. It has been previously shown that XLT patients have reduced levels of WASp and correspondingly reduced numbers of podosomes (Linder et al., 2003). Normal cells were found to have an average of 30.9 podosomes per cell compared to 15 in WASp KO cells (**Figure 4.6**). Only cells that have podosomes present were included in this average. WASp KO dendritic cells transduced with CEWW had an average of 37.7 and with SEWW had an average of 36.6 showing that the transduced cells were reconstituted to levels that were above normal (**Figure 4.6**).

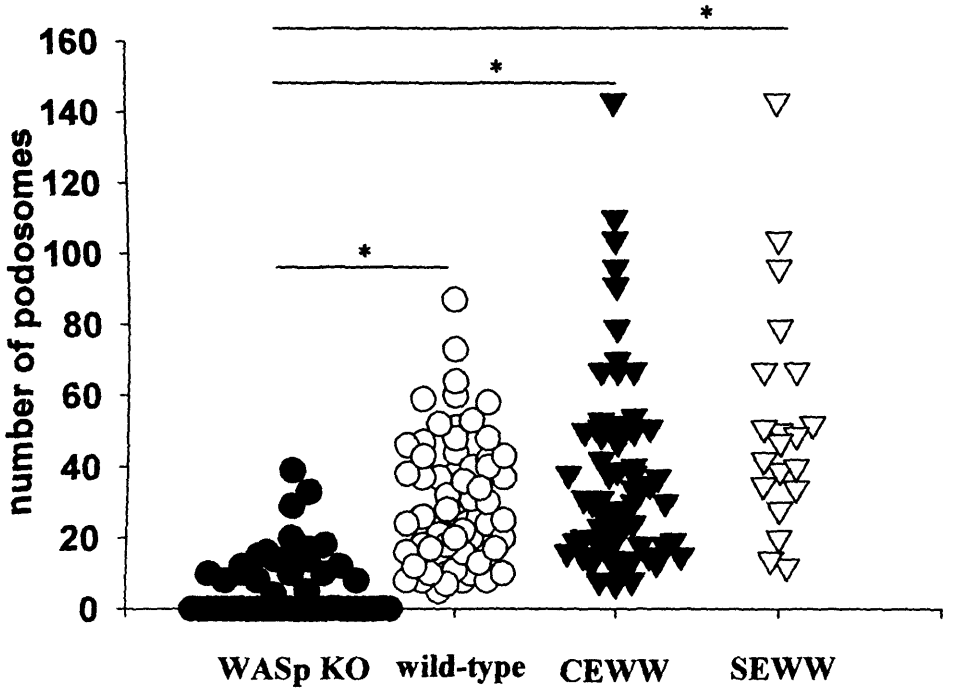


Figure 4.6 Quality of reconstitution of podosomes in dendritic cells
Bone marrow derived dendritic cells transduced with EGFP-WASp were stained with phalloidin to visualise F-actin and therefore podosomes. Positive transduced cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. CEWW = CMV EGFP-WASP WPRE, SEWW = SFFV EGFP-WASp WPRE. * $p<0.001$ students T test

There were some cells transduced with either CEWW or SEWW that contained very high numbers of podosomes that were not seen in normal cells, which may be the reason for this high average. When the distribution of podosome number was calculated based upon the percentage within specific groups it was found to be almost identical to that seen in normal cells with little or no skewing of the populations towards high or low numbers of podosomes (**Figure 4.7**).

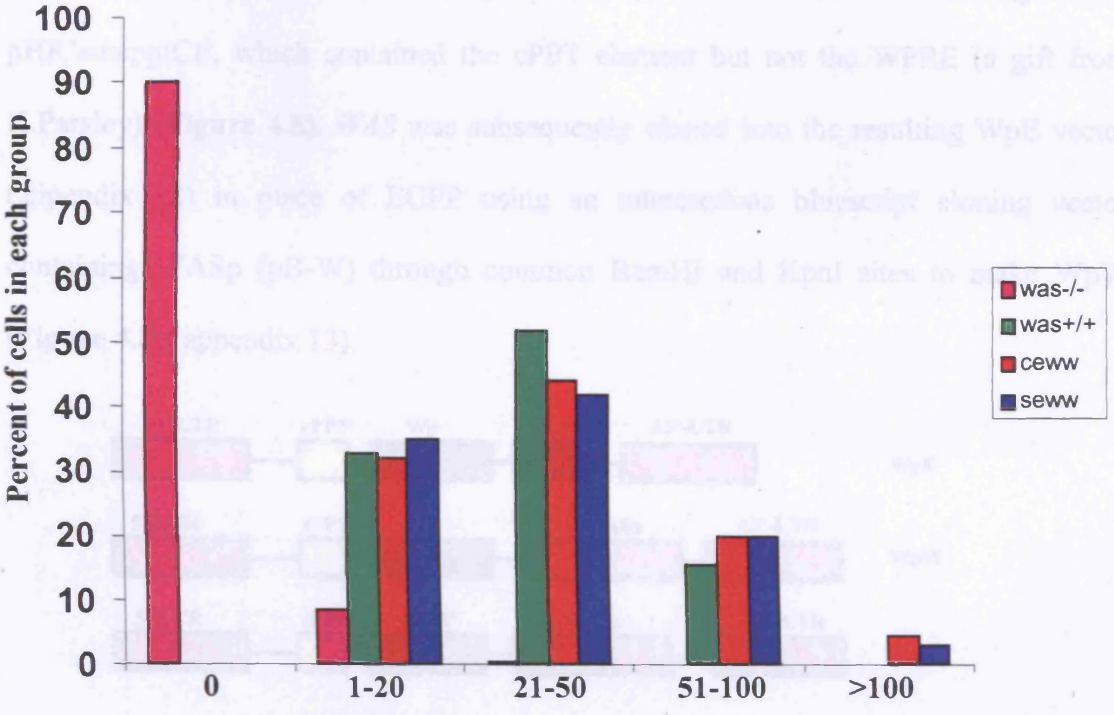


Figure 4.7 Distribution of podosome number per cell
Dendritic cells were transduced with EGFP-WASp (CEWW or SEWW) and were scored into groups according to podosome content. For transduced and WASp+/+ dendritic cells only the cells with podosomes were included whereas all cells are included for WASp-/- cells.

4.2.2 Cloning of Putative Endogenous Wiskott Promoter Sequence

A 500bp sequence upstream of the WAS start codon is one sequence shown in luciferase reporter assays to have good promoter activity in haematopoietic cell lines (Hagemann and Kwan, 1999). This endogenous promoter sequence was amplified by

PCR from genomic DNA derived from Ficolled normal peripheral blood lymphocytes. As well as amplifying the endogenous wiskot promoter these primers incorporated EcoRI and BamHI restriction sites 5' and 3' of the 500bp sequence for use in subsequent cloning. A band corresponding to 500bp was excised from an agarose gel and purified. The band was gel extracted. Sequencing confirmed the correct sequence was amplified (appendix 11). The EcoRI and BamHI sites were used to replace the SFFV LTR with the endogenous regulatory sequence in an EGFP containing vector, pHR'sincpptCE, which contained the cPPT element but not the WPRE (a gift from K.Parsley) (**Figure 4.8**). *WAS* was subsequently cloned into the resulting WpE vector (appendix 12) in place of EGFP using an intermediate bluescript cloning vector containing WASp (pB-W) through common BamHI and KpnI sites to make WpW (**Figure 4.8**)(appendix 13).

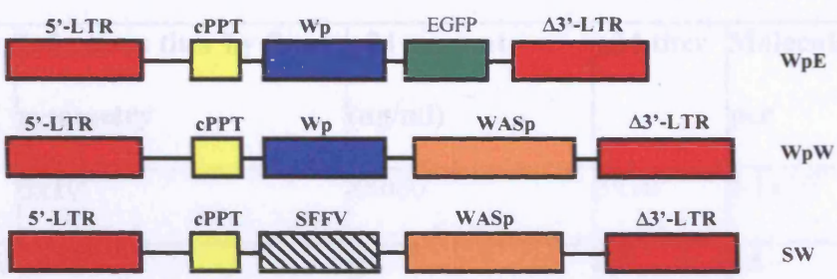


Figure 4.8 Cloning of lentiviral vectors containing an endogenous promoter
Cartoon depictions of lentiviral constructs containing a 500bp endogenous promoter sequence (Wp) and a construct with an SFFV LTR with WASp only. Also shown are the major features.

4.2.2.1 Titration of lentiviral vectors containing endogenous promoter sequences and WASp

Titration of virus particles is easiest when there is a direct way to visualise the percentage of transduced cells e.g. EGFP, and was part of the rationale behind creating a fusion protein of EGFP and WASp. When WASp was the transgene there was no

reliable way of determining the percent of transduced cells and therefore a titer. In addition it was found that the level of EGFP expression obtained from the endogenous WASp promoter sequence was not as high as that observed from cells transduced with CEW or SEW, although this is probably in part due to the lack of the WPRE. Therefore other reliable methods of titration were used. One such method is calculation of the concentration of HIV-1 capsid protein p24 in the viral supernatant. It has been calculated that 1pg of p24 equates to 10^4 physical particles with 10-100 transducing units per pg of p24 allowing the total number of particles to be calculated (http://tronolab.com/TUvsp24.php). **Table 4.1** shows representative data comparing infectious titres where applicable and the values obtained by p24 ELISA. It appears that p24 ELISA titres give values that are about 10 fold higher than those observed by infectious titration methods suggesting that there are uninfectious particles present.

Sample	Infectious titer by flow cytometry	p24 amount (ng/ml)	p24 titer	Molecular titer by pcr
SEW	$5x10^8$	46000	$5x10^9$	$4.1x10^9$
CEWW	$2x10^8$	nd	nd	nd
SEWW	$1.75x10^8$	19500	$1.9x10^9$	$4.9x10^9$
SWW	nd	87100	$8.7x10^9$	$8.7x10^9$
WpE	$2.8x10^8$	75300	$7.5x10^9$	$1x10^{10}$
WpW	nd	450000	$4.5x10^{10}$	$4.6x10^9$

Table 4.1 Comparison of titration methods
Viral titers were calculated by infectious titer on a permissive cell line judged by the percent positive for the transgene, p24 ELISA, where the amount of capsid protein relates to virus quantities or quantitative pcr, where total copies of integrated viral genomes is calculated compared against a known standard. nd = not determined

An alternative method used to titre virus was using quantitative real time PCR. Although titres can be obtained direct from vector supernatant RNA (Lizee et al., 2003) we calculated the titer based upon the number of integrated proviral DNA. This is because they are least likely to be influenced by factors such as empty or defective particles (a problem when using p24 ELISA), or expression levels (a problem for infectious titres and supernatant RNA) and has been shown to be reproducible and reliable compared to other methods (Sastry et al., 2002b).

Using plasmid DNA to generate standard curves for viral genomes and β-actin (to determine cell number), infected cells had their genomic DNA purified, followed by real-time quantitative PCR analysis to determine the copy number within the cell population. Thus the copy number multiplied by the dilution factor of the virus upon infection multiplied by the cells infected gives a titer. When the infectious titer and Q-PCR titer were compared there was a consistent 10-fold increase in Q-PCR titer (**Table 4.1**). This result was similar to that previously reported (Sastry et al., 2002c) therefore molecular titers were used for vectors containing WASp alone or where endogenous promoters were used to allow accurate comparison between vectors.

4.2.2.2 Reconstitution of podosomes using vectors containing an endogenous promoter

Bone marrow derived dendritic cells were plated onto coverslips and infected with WpE WpW and SW at an MOI of 1000 (molecular titer) and incubated for 72 hours. The cells were then fixed and counterstained with phalloidin and vinculin to enable scoring of cells with podosomes as described previously. As expected cells transduced with WpE did not restore podosomes. Cells transduced with WASp from either WpW or SW

however had podosomes restored to 15% and 25% respectively (**Figure 4.9**), which is up to half of normal levels. Counting the number of podosomes per cell assessed the quality of restoration. There was again high quality reconstitution. Normal cells were found to have an average of 30.9 podosomes per cell compared to 15 in WASp KO dendritic cells, when only positive cells were included (**Figure 4.10**).

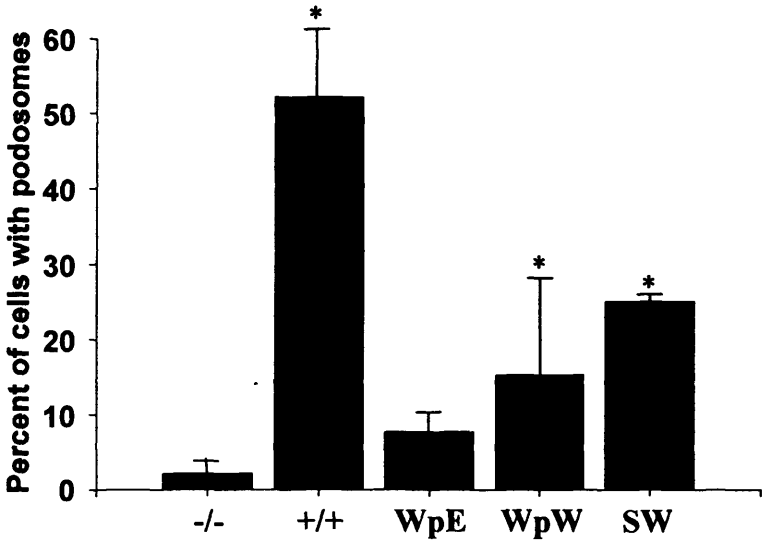


Figure 4.9 Reconstitution of podosomes
Bone marrow derived dendritic cells were seeded onto glass coverslips and infected at an MOI of 1000 determined by molecular titer with EGFP (WpE) or WASp (WpW, SW). The cells were then fixed and permeabilised before staining with rhodamine phalloidin to visualise F actin. Confocal images of the cells were scored for the presence or absence of podosomes on the basal surface. * $p<0.005$ students T test

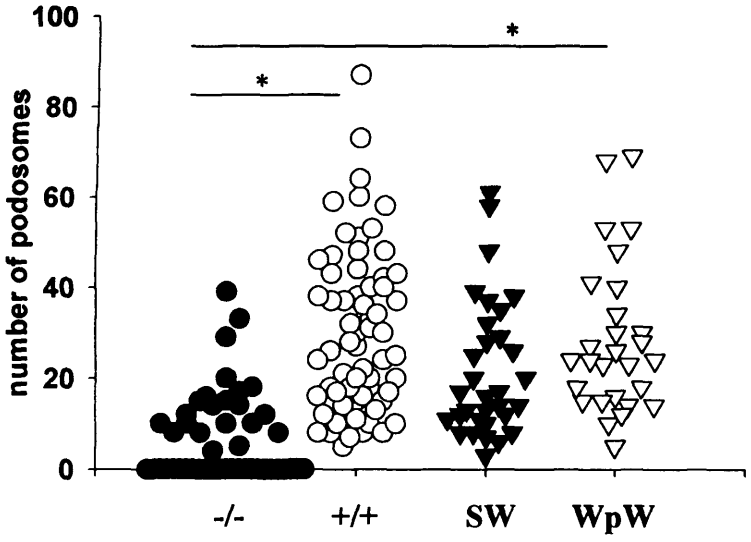


Figure 4.10 Quality of reconstitution of podosomes in dendritic cells
Bone marrow derived dendritic cells transduced with WASp were stained with phalloidin to visualise F-actin and therefore podosomes. Positive transduced cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. WpW = endogenous promoter WASp, SW = SFFV WASp. * $p<0.001$ students T test compared to WAS KO

Cells transduced with SW and WpW had an average of 21.5 and 28.6 podosomes per cell which is higher than WASp KO cells and in the case of WpW transduced cells is approaching normal levels. Again the distribution of podosomes within the dendritic cell population was measured by calculating the percentage in defined groups. Cells transduced with SW had a higher proportion of cells with lower amounts of podosomes when compared to normal cells, which is suggested by the lower average podosome number, whereas cells transduced with WpW had a near normal distribution again reflecting the near normal average podosome number (**Figure 4.11**) The podosomes seen by confocal microscopy in transduced cells were often present at leading edges and either had a ring of vinculin surrounding the actin core, or vinculin in close proximity to actin, showing correct podosome assembly for both WpW and SW transduced cells (**Figure 4.12**).

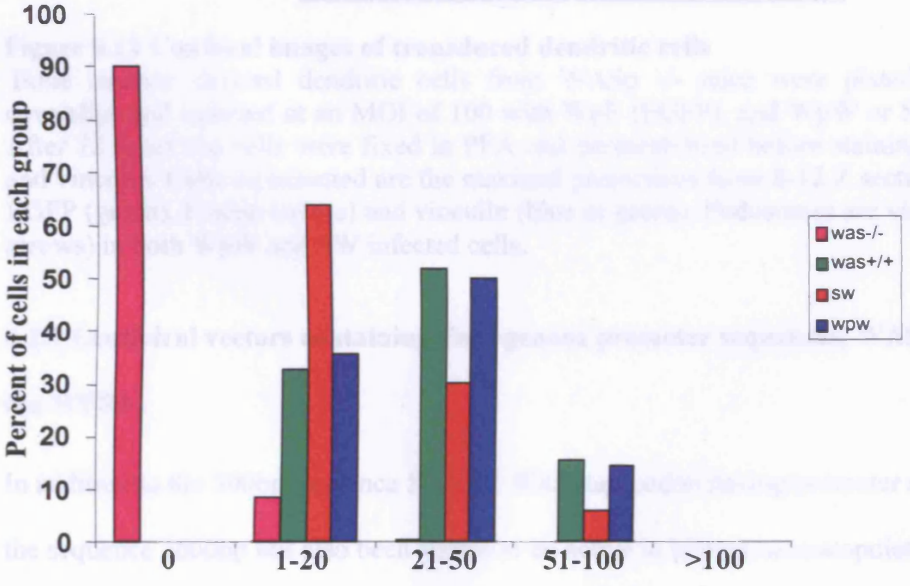


Figure 4.11 Distribution of podosome number per cell
Dendritic cells were transduced with WASp (WpW or SW) and were scored into groups according to podosome content. For transduced and WASp+/+ dendritic cells only the cells with podosomes were included whereas all cells are included for WASp-/- cells.

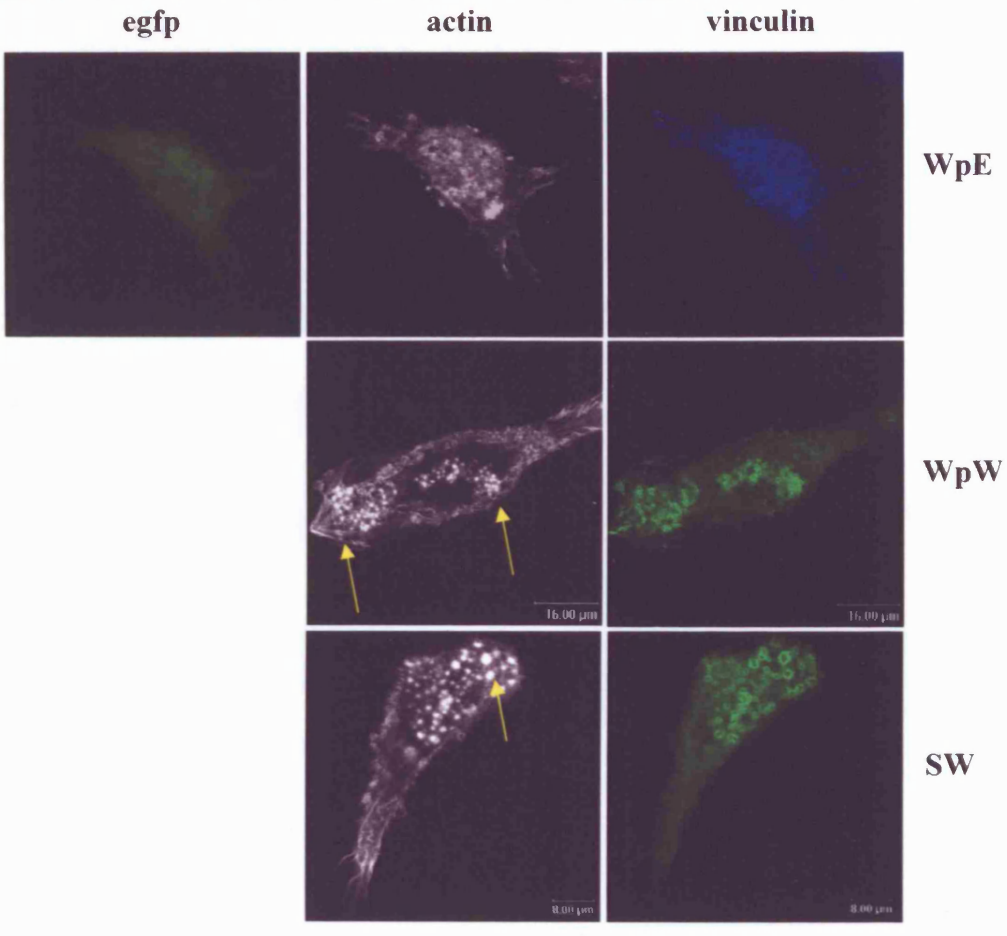


Figure 4.12 Confocal images of transduced dendritic cells
Bone marrow derived dendritic cells from WASp -/- mice were plated onto glass coverslips and infected at an MOI of 100 with WpE (EGFP), and WpW or SW (WASp). After 72 hours the cells were fixed in PFA and permeabilised before staining for F-actin and vinculin. Cells represented are the maximal projections from 8-12 Z sections showing EGFP (green), F-actin (white) and vinculin (blue or green). Podosomes are visible (yellow arrows) in both WpW and SW infected cells.

4.2.3 Lentiviral vectors containing Endogenous promoter sequences, WASp and the WPRE.

In addition to the 500bp sequence 5' of the *WAS* start codon having promoter activity the sequence 1600bp has also been shown to be active in human haematopoietic cells (Petrella et al., 1998) as well as having 2 conserved regions between the proximal and distal promoter sequences (Ann Galy, personal communication). Both the 500bp and 1600bp sequences were cloned into a pRRL based lentiviral vector containing *WAS*, which included the mutated WPRE sequence. These vectors were used in transduction

experiments in conjunction with an SFFV 5'LTR containing vector (**Figure 4.13**). All 3 vectors were a gift from Ann Galy, Genethon, France and were titered based upon a molecular quantitative PCR method.

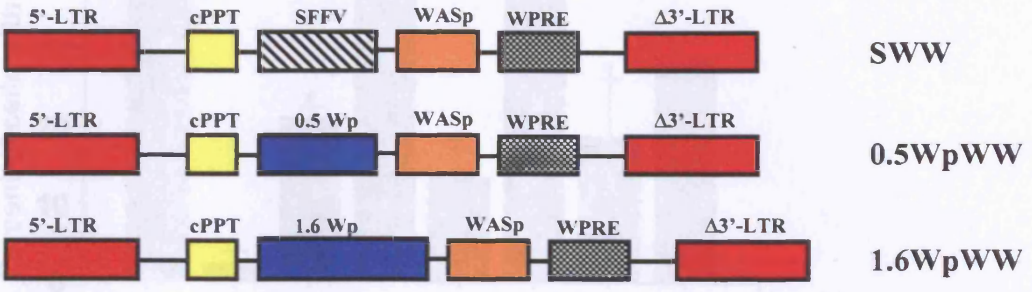


Figure 4.13 Cloning of lentiviral vectors containing various internal promoters
Cartoon depictions of lentiviral constructs containing WASp alone that contain the WPRE. Three different promoters were cloned 500bp endogenous promoter sequence (0.5Wp), a 1600bp endogenous promoter sequence (1.6Wp) and a construct with an SFFV LTR. Also shown are the major features present in the backbone.

These vectors ability to reconstitute podosomes in murine dendritic cells was used to determine which vector is the most efficient and therefore most suitable for use in a clinical trial. Dendritic cells were infected at a low MOI (100) and a high MOI (1000) and the number of cells with podosomes reconstituted counted. Normal dendritic cells were 52% positive for podosomes compared to >5% in WASp KO cells (**Figure 4.14**). There was an increase in the number of cells with podosomes when cells were infected with SWW, 0.5WpWW and 1.6WpWW at an MOI of 100 to 21.1%, 13.7% and 18.6%, respectively (**Figure 4.14**). Podosome recovery was increased further by an increase in the MOI to 1000. Cells infected with SWW, 0.5WpWW and 1.6WpWW all showed an increase in the percentage recovery to 36.9%, 40.2% and 29%, respectively (**Figure 4.14**).

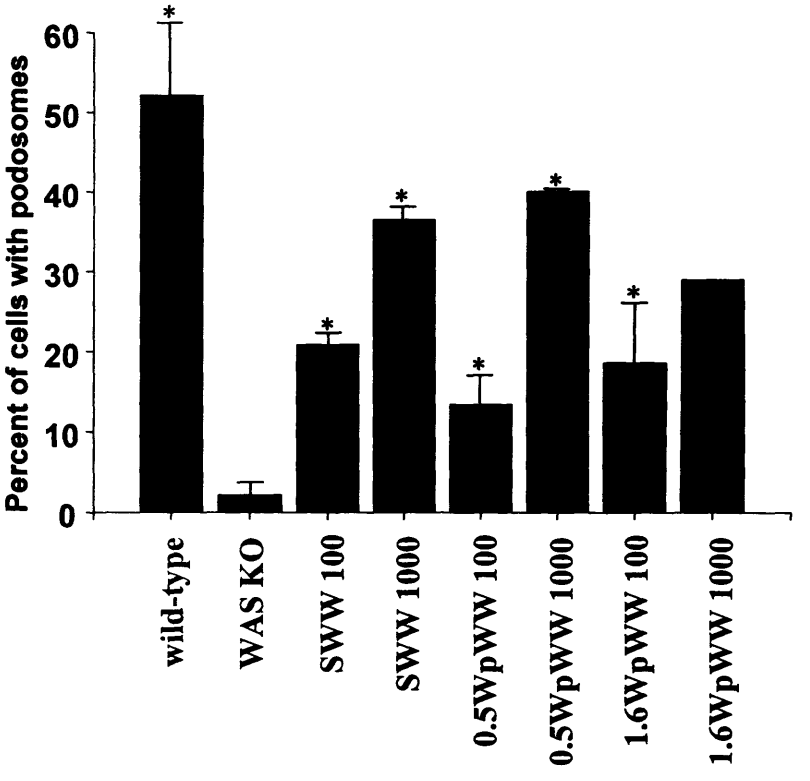


Figure 4.14 Reconstitution of podosomes
Bone marrow derived dendritic cells were seeded onto glass coverslips and infected at an MOI of 100 and 1000 determined by molecular titer with WASp under the control of a 500bp endogenous promoter sequence, a 1600bp endogenous promoter sequence and an SFFV LTR. The cells were then fixed and permeabilised before staining with rhodamine phalloidin to visualise F actin. Confocal images of the cells were scored for the presence or absence of podosomes on the basal surface. 100-200 cells were scored. Error bars shown are +1 SD * $p<0.002$ students T test compared to WAS KO.

4.2.3.1 Quality of Reconstitution

When the quality of reconstitution, determined by the number of podosomes per cell was determined there was again an increase in quality with a corresponding increase in MOI. The average number of podosomes per cell for normal cells was 31.7 compared to 15 for WASp KO cells, in those cells that contained podosomes. The number of podosomes per cell increased from 24.6 to 30.7 in SWW infected cells, remained relatively similar at 24 and 25 in 0.5WpWW cells and increased from 19.3 to 30.1 in 1.6WpWW infected cells (**Figure 4.15**). Confocal images, used to score the presence and number of podosomes, revealed podosomes were present in normal cells and WASp

KO cells transduced with WASp but not EGFP (**Figure 4.16**). No vinculin staining was performed on these samples.

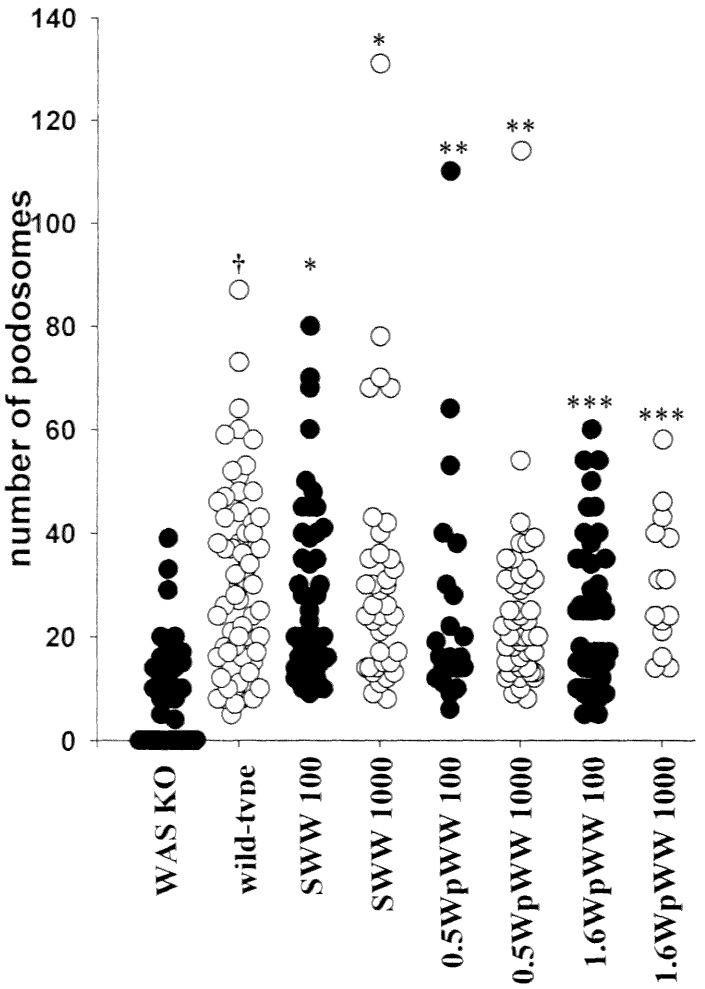


Figure 4.15 Quality of reconstitution of podosomes in dendritic cells
Bone marrow derived dendritic cells transduced with WASp were stained with phalloidin to visualise F-actin and therefore podosomes. Positive transduced cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. 0.5WpWW = 500bp endogenous promoter WASp, 1.6WpWW = 1600bp endogenous promoter WASp, SWW = SFFV WASp. † $p<0.001$, * $p<0.05$, ** $p< 0.5$, *** $p< 0.01$ students T test compared to values obtained for WAS KO.

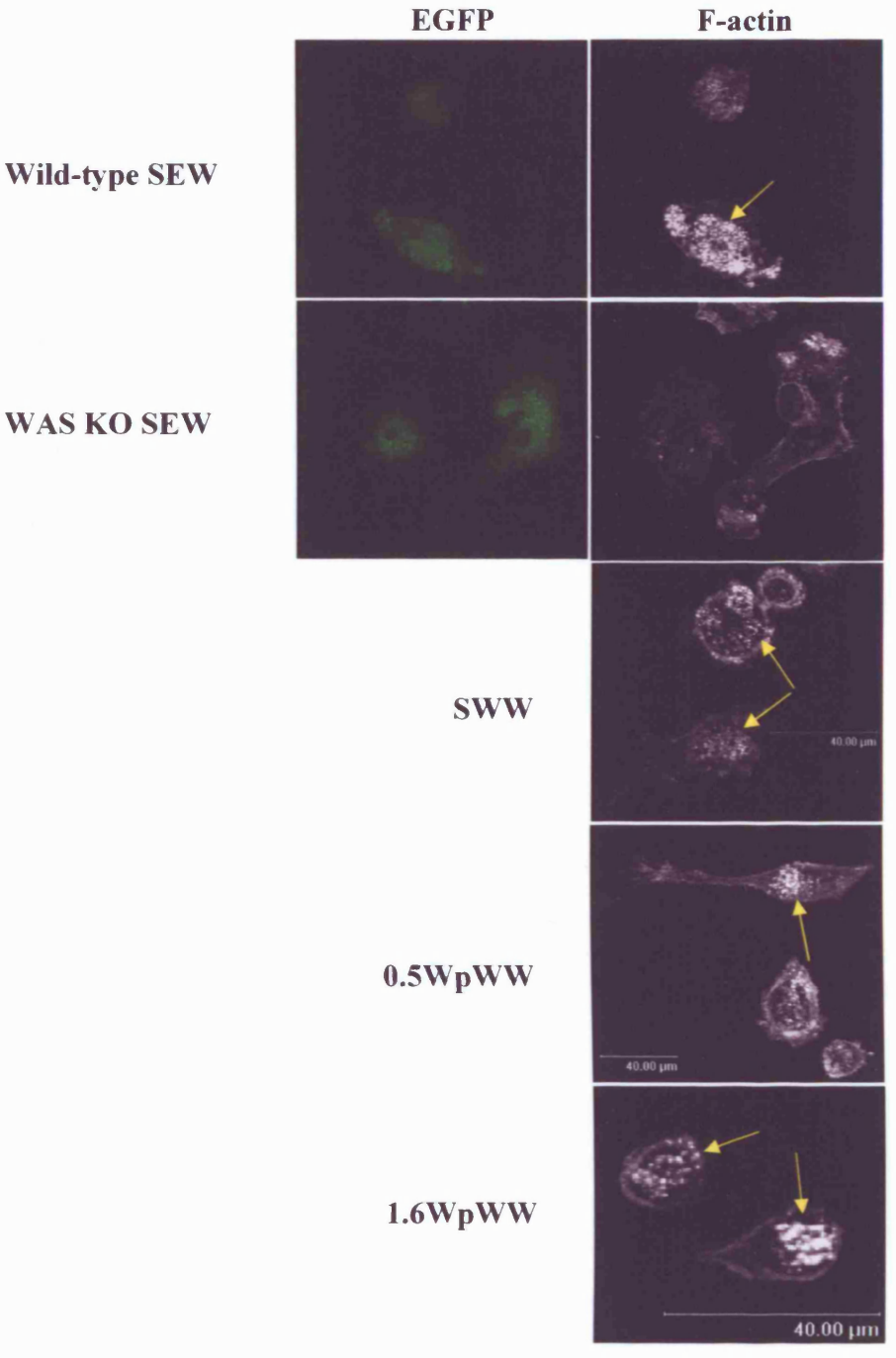


Figure 4.16 Confocal images of transduced dendritic cells
Bone marrow derived dendritic cells from WASp -/- mice were plated onto glass coverslips and infected at an MOI of 1000 with 0.5WpWW, 1.6WpWW or SWW (WASp). After 72 hours the cells were fixed in PFA and permeabilised before staining for F-actin. Cells represented are the maximal projections from 8-12 Z sections showing EGFP (green) and F-actin (white). Podosomes are visible (yellow arrows) in all infected cells.

which were similar in appearance to that observed with transient transfections in dendritic cells (Chapter 3). Confocal images revealed co-localisation of actin and EGFP-WASp in podosomes of transduced cells that was not observed in EGFP transduced normal or WASp null cells; although no vinculin staining was performed on these samples. The podosome cluster was often present at the leading edge of the cell reminiscent of the position in normal cells (**Figure 4.19**).

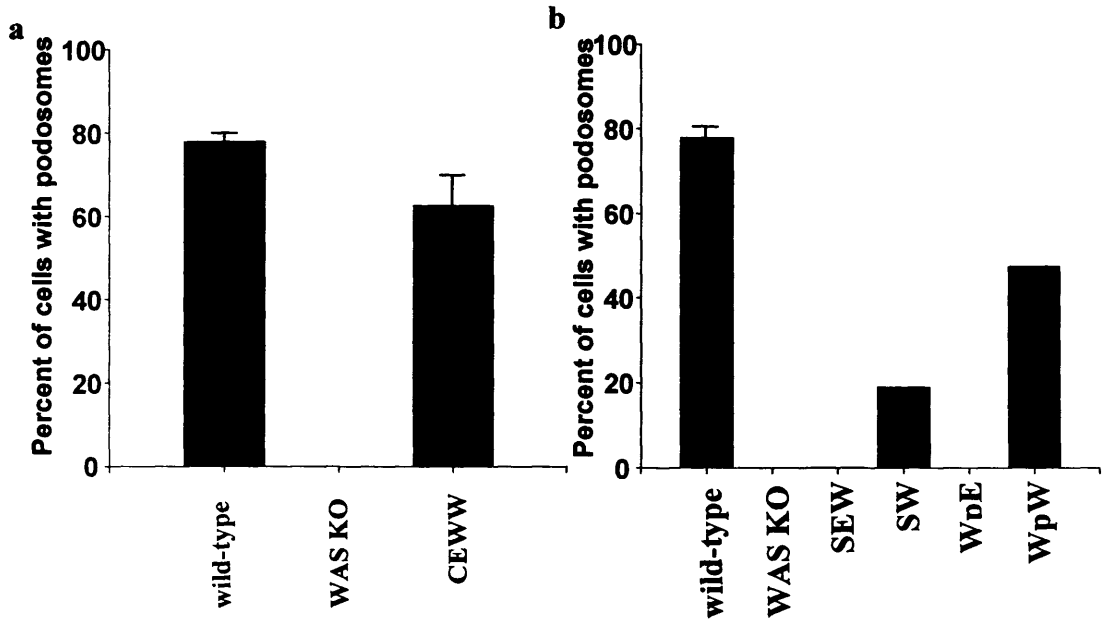


Figure 4.18 Podosome recovery in primary human macrophages
Primary human macrophages were seeded onto glass infected at an MOI of 100 with **a)** EGFP-WASp (CEWW) or **b)** EGFP (SEW and WpE) or WASp (SW and WpW). The cells were fixed and stained with phalloidin to reveal F-actin. Cells were scored for the presence or absence of podosomes. Error bars shown are +1 SD

WASp null cells were also infected with vectors containing WASp alone under the control of an SFFV LTR or the 500bp endogenous WASp promoter at a molecular titer of 1000. Cells transduced with SW and WpW restored podosomes in 19% and 47.5% of cells respectively (**Figure 4.18b**). These cells also had their podosomes often present at the leading edge mimicking that seen in normal macrophages (**Figure 4.19**).

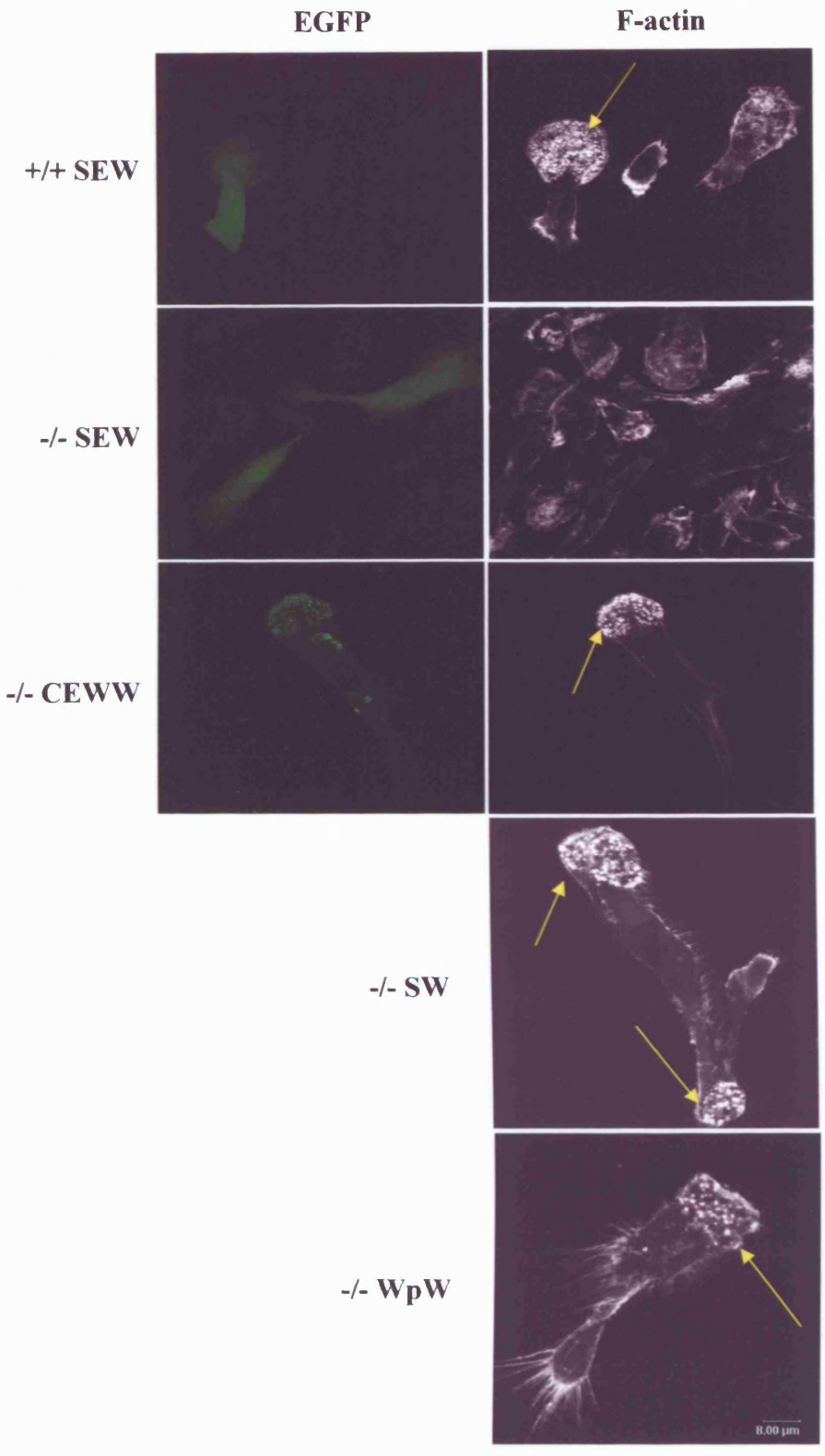


Figure 4.19 Confocal images of transduced macrophages
Peripheral blood derived macrophages from WASp null patients were plated onto glass coverslips and infected at an MOI of 100 with EGFP (SEW), EGFP-WASp (CEWW) or WASp (SW+WpW). After 72 hours the cells were fixed in PFA and permeabilised before staining for F-actin. Cells represented are the maximal projections from 8-12 Z sections showing EGFP and EGFP-WASp (green) and F-actin (white). Podosomes are visible (yellow arrows) in normal cells and in WASp null cells infected with EGFP-WASp and WASp.

4.2.4.1 Quality of Podosome Reconstitution

Again the quality of reconstitution was measured by counting the number of podosomes in reconstituted cells and comparing to normal. The average number of podosomes per cell in normal controls is 40.4 whereas WASp null cells with podosomes were greatly reduced at an average of 7.2 podosomes per cell (**Figure 4.20**). Macrophages infected with CEWW had an average number of podosomes of 56.6 showing there was high quality reconstitution that is similar to that observed in normal cells (**Figure 4.20a**). Cells transduced with WASp alone also showed high quality reconstitution. The average number of podosomes per cell was 85.3 for SW and 26.4 for WpW transduced cells (**Figure 4.20b**), although the very low sample number for SW prevents any meaningful conclusions being drawn.

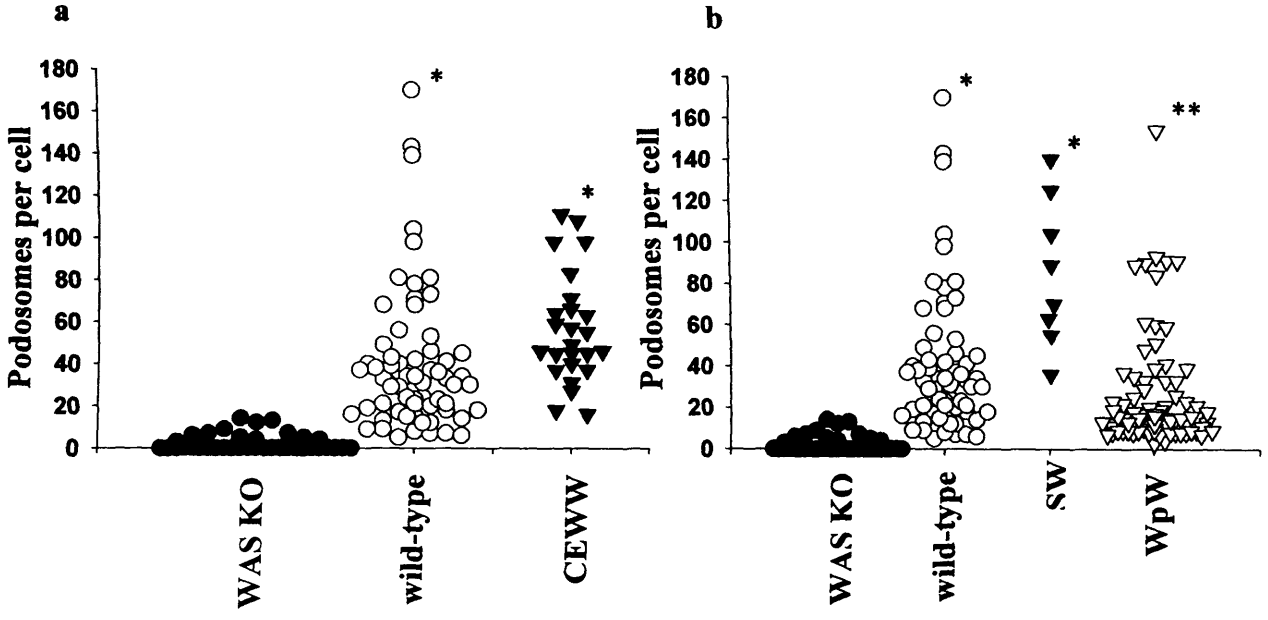


Figure 4.20 Quality of reconstitution of podosomes in macrophages
Peripheral blood derived macrophages transduced with a) EGFP-WASp and b) WASp were stained with phalloidin to visualise F-actin and therefore podosomes. Positive transduced cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. CEWW = CMV EGFP-WASp, WpW = 500bp endogenous promoter WASp, SW = SFFV WASp. * $p < 0.001$, ** $p < 0.02$ students T test

The distribution of podosomes was measured by calculating the percentage within defined groups to determine if there is a normal range of podosome number. Cells transduced with CEWW had a near normal distribution with an increase in the 51-100 group, probably accounting for the increase in the average number of podosomes per cell (**Figure 4.21**). Cells transduced with SW had a distribution that was higher than normal showing most of the cells in the two highest groups, but the low sample number negates the significance of this (**Figure 4.21**). The distribution of podosomes in cells transduced with WpW showed an increase in cells with low numbers of podosomes compared to normal, indicative of a lower level of expression (**Figure 4.21**).

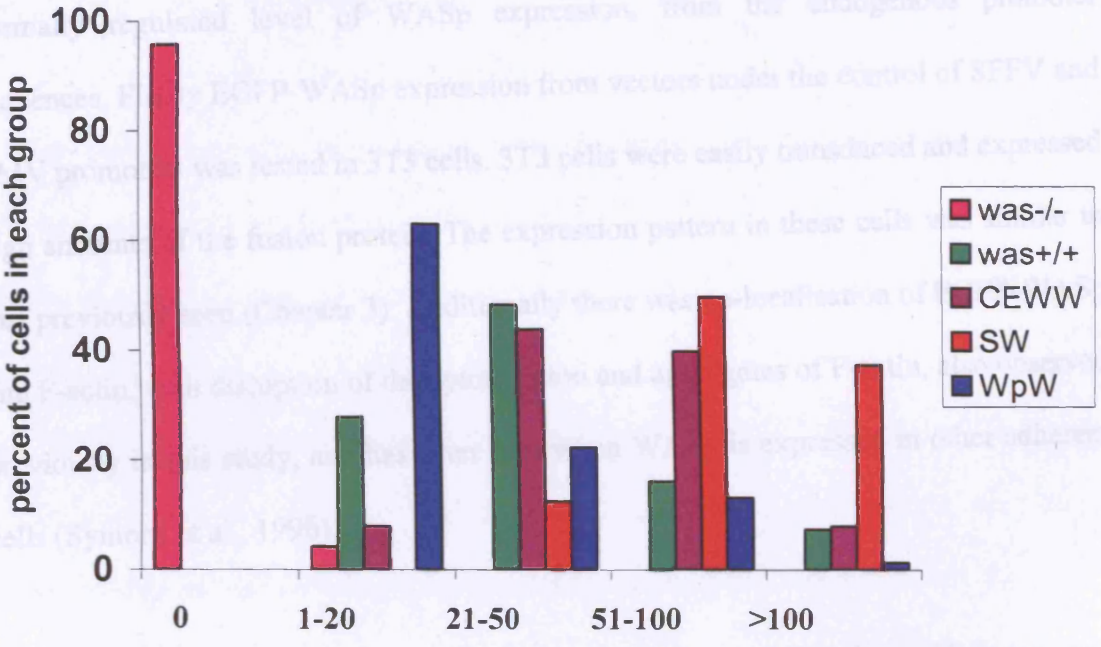


Figure 4.21 Distribution of Podosome Number per cell
Peripheral blood derived macrophages were transduced with a) EGFP-WASp (CEWW) or b), c) WASp (SW and WpW) and were scored into groups according to podosome content. For transduced and WASp+/+ macrophages cells only the cells with podosomes were included whereas all cells are included for WASp-/- cells.

4.3 Discussion

The aims of this chapter were to develop VSV-G pseudotyped self-inactivating HIV-1 derived lentiviral vectors for use in reconstitution *in vitro* of primary murine WASp KO and human WASp null cells. The data obtained could then be used to help select a vector suitable for use in an *in vivo* gene therapy model. Vectors were developed containing EGFP, EGFP-WASp or WASp as the transgene, under the internal control of either strong viral promoters or putative endogenous promoter sequences with or without the WPRE sequence. This would enable a comparison between the constitutively active promoters and high level WASp expression, and a possibly more normally regulated level of WASp expression, from the endogenous promoter sequences. Firstly EGFP-WASp expression from vectors under the control of SFFV and CMV promoters was tested in 3T3 cells. 3T3 cells were easily transduced and expressed high amounts of the fusion protein. The expression pattern in these cells was similar to that previously seen (Chapter 3). Additionally there was co-localisation of EGFP-WASp and F-actin, with disruption of the cytoskeleton and aggregates of F-actin, also observed previously in this study, and has been seen when WASp is expressed in other adherent cells (Symons et al., 1996).

Vectors containing viral promoters for EGFP-WASp expression allowed infection and high-level transduction of murine primary bone marrow derived dendritic cells (**Figure 4.4**). These cells had their podosomes restored to 60-65% of normal showing that upwards of 80% of transduced cells were able to restore podosomes. These cells had podosomes at leading edges, had F-actin and EGFP-WASp co-localised within the podosome and although often no ring of vinculin was visible there was definite co-localisation, suggesting normal podosome assembly. Similar levels of podosome

numbers per cell with a normal distribution were obtained with both viral promoters (**Figures 4.6 +4.7**) suggesting a high level of reconstitution.

Although obtaining viral titers calculated from infection efficiency is a widely used and reproducible method of titering, it was not possible to use this method for viral vectors containing WASp alone due to unreliable intracellular WASp staining. In addition with endogenous promoters there was no expression in non-haematopoietic cells (Martin et al., 2005). A molecular titre, obtained by real-time quantitative PCR upon infected cells, was the best method for obtaining a reliable viral titer. Although titers obtained by this method are generally higher than infectious titres (**Table 4.1**) (Sastry et al., 2002a) and are subject to a reliable standard curve, a direct comparison between vectors is obtainable and allows similar quantities of virus to be added to cells, therefore allowing accurate comparisons between vectors.

Putative endogenous promoter containing vectors allowed reconstitution of WASp KO dendritic cells. When the WPRE was not present the level of reconstitution was lower with the 500bp endogenous promoter compared to the SFFV LTR. The quality of the reconstitution however was better and had a more normal distribution. When the WPRE was introduced and the 1600bp endogenous promoter was also compared there was little difference between the promoters ability to reconstitute podosomes. Similarly there was little difference in the quality of reconstitution and distribution of podosomes in murine dendritic cells.

When human primary macrophages were infected they too were able to have their podosomes restored when either viral promoters or endogenous promoter sequences were used. Furthermore the podosomes formed were very similar to those observed in normal macrophages being formed at leading edges and showing co-localisation of F-

actin and EGFP-WASp. Although there is very high sequence homology between the murine sequence 5' of the start codon of WASp and the human sequence, the use of the 500bp endogenous promoter lead to podosome numbers that were lower than that seen in normal which was not observed in murine dendritic cells. It could be a possibility that there are other sequences, such as those found in the 1600bp promoter that are important for either cell specific expression or for species specific expression as there is little homology further upstream of the 500bp fragment used. This was also without the inclusion of the WPRE, which was shown to improve the quality of reconstitution in murine dendritic cells (**Figure 4.14**) compared to vectors lacking the WPRE (**Figure 4.9**).

Endogenous promoters may be a suitable alternative to viral promoters for use in a clinical gene therapy vector, where good efficient transduction and expression is obtained and is comparable to the levels obtained from viral promoters. There have been some problems associated with the use of viral LTRS namely the risk of insertional mutagenesis. It has been shown in animal models that multiple insertional events can lead to leukaemia (Modlich et al., 2005) (Li et al., 2002) where high doses of virus are used and where there are multiple insertional events around proto-oncogenes and signalling genes. In addition the leukaemia associated with the SCID-X1 trial in France was associated with insertional mutagenesis of the LMO-2 gene (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Lentiviral vectors, as do some retroviral vectors have the ability to insert multiple copies into each cell (Woods et al., 2003) increasing the risk of insertional mutagenesis. The use of self-inactivating vectors reduces this risk as there is a lack of enhancer elements in the integrated viral LTR (Zufferey et al., 1998) as well as reducing the possibility of replication competent retrovirus. As WASp is normally highly restricted to the haematopoietic lineage the use

of endogenous promoter sequences (either the 500bp or 1600bp fragment) in a viral vector may reduce the potential risk of complications from abnormal regulation of cellular expression.

This data represents the first case of WAS reconstitution in myeloid cells *in vitro* using lentiviral vectors. Previous retroviral *in vitro* work has focused on B cell lymphoblastic cell lines (Candotti et al., 1999) and particularly primary T cells with both retroviral and lentiviral vectors (Wada et al., 2002; Strom et al., 2003b; Strom et al., 2003a; Dupre et al., 2004).

Although the endogenous promoter sequences work well in the myeloid lineages *in vitro* it remains to be seen if they are capable of expression in pluripotent stem cells and allow expression in multiple lineages of the immune system in an *in vivo* setting.

CHAPTER 5

RECONSTITUTION OF A MURINE MODEL OF WAS USING LENTIVIRAL VECTORS

5.1 Introduction

We have made lentiviral particles with different internal promoters capable of transducing cell lines and primary cells *in vitro* and have shown successful reconstitution of dendritic cells *in vitro* with WASp using these lentiviral vectors (Chapter 4). The next step was to test the vectors ability to reconstitute a WAS KO murine model. This involves transduction *ex vivo* of murine stem cells followed by injection into lethally irradiated recipients to restore a normal phenotype again using podosome recovery as a tool to measure transduction. Using a modified retroviral protocol (Chapter 3), lineage negative cells were transduced *ex vivo* and transplanted into lethally irradiated recipients (Biffi et al., 2004). In this way we were able to optimise a lentiviral infection protocol capable of high levels of transduction. It was hoped that an improved level of transduction of murine stem cells could be obtained with lentiviral vectors over that previously seen with oncoretroviral vectors and lead to stable long term recovery of WAS.

Reconstitution of podosomes and number of podosomes per cell were used as a proxy measurement of efficiency of transduction in addition to the levels of EGFP (where present). Reconstitution in T cells was also measured by recovery of a proliferation response when stimulated.

5.2 Results

5.2.1 Reconstitution of WAS KO mouse model Using CMV and SFFV containing vectors

The basic protocol used in the generation of an in vivo model is outlined in **Figure 5.1**. Briefly bone marrow derived lineage negative murine stem cells were harvested from the bone marrow, infected *ex vivo* in the presence of cytokines overnight and infused into a lethally irradiated recipient to allow the donor cells to repopulate the haematopoietic system. After a period of incubation, ranging from 6 weeks to 5 months, the mice were sacrificed and the lymphoid organs analysed for the presence of transgene and for reconstitution of function.

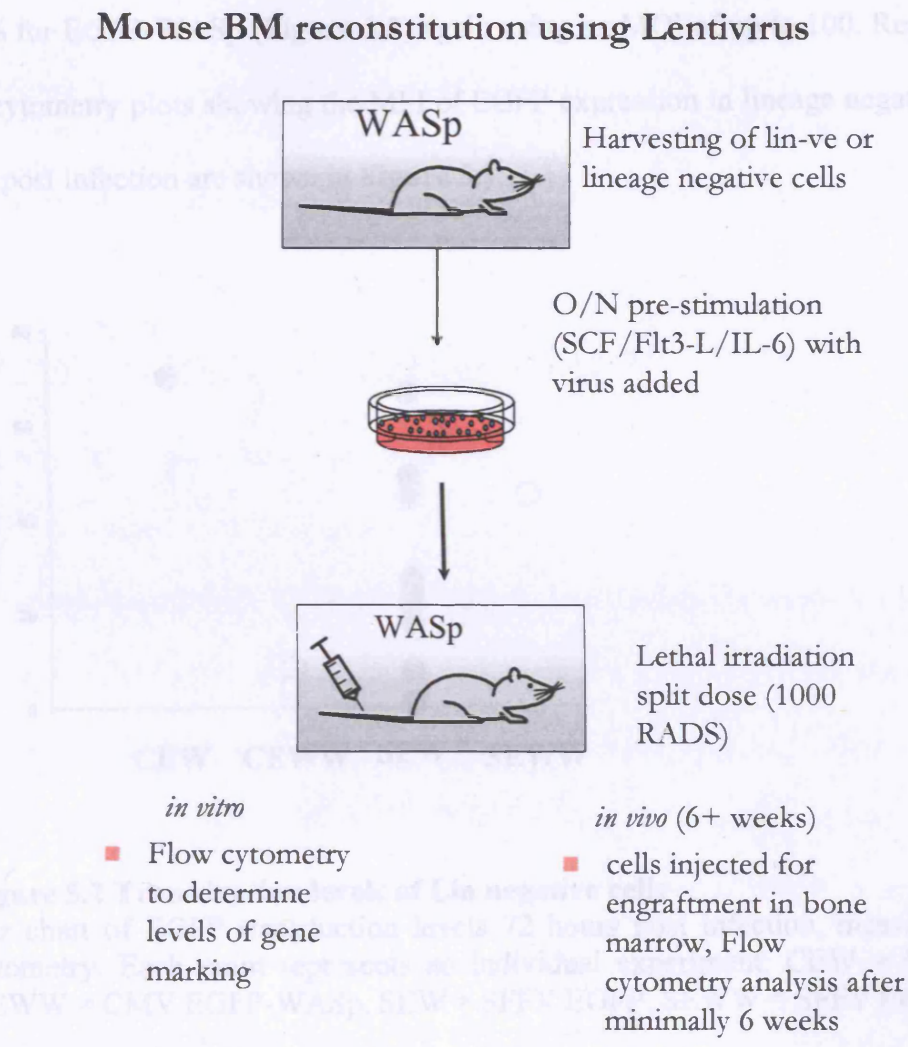


Figure 5.1 *Ex vivo* Transduction Protocol
Cartoon depiction of the transduction protocol. Lineage negative cells were harvested and cultured with virus overnight in the presence of cytokines. The cells were washed and infused back into lethally irradiated recipients and allowed to engraft for at least 6 weeks before mice are sacrificed. A sample was kept in culture for 72 hours to be analysed by flow cytometry for EGFP levels.

5.2.2.1 Transduction efficiency of Lineage Negative stem cells

There was successful gene marking in lineage negative cells when EGFP and EGFP-WASp containing vectors were used (**Figure 5.2**). When vectors containing the CMV promoter were used at an MOI of 100 the efficiency of transduction was up to 70% for EGFP and 10% for EGFP-WASp, determined by flow cytometry. The efficiency of transduction was improved when vectors containing an SFFV LTR as the internal promoter were used. The efficiency of transduction ranged from 1-66% for EGFP and 1-45% for EGFP-WASp (**Figure 5.2**) again using an MOI of up to 100. Representative flow cytometry plots showing the MFI of EGFP expression in lineage negative cells 72 hours post infection are shown in **Figure 5.3**.

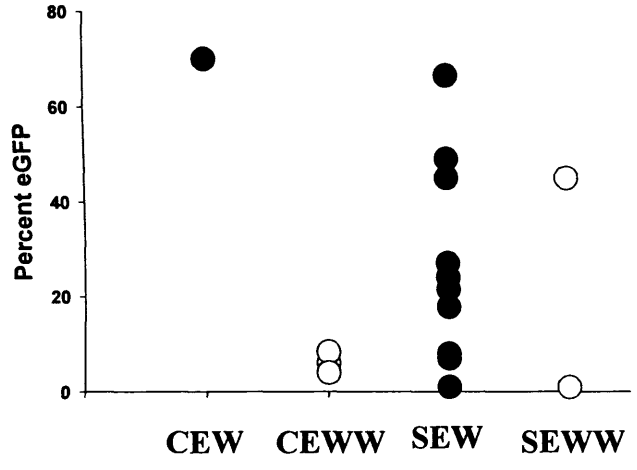


Figure 5.2 Transduction levels of Lin negative cells
Bar chart of EGFP transduction levels 72 hours post infection, measured by flow cytometry. Each point represents an individual experiment. CEW = CMV EGFP, CEWW = CMV EGFP-WASp, SEW = SFFV EGFP, SEWW = SFFV EGFP-WASp

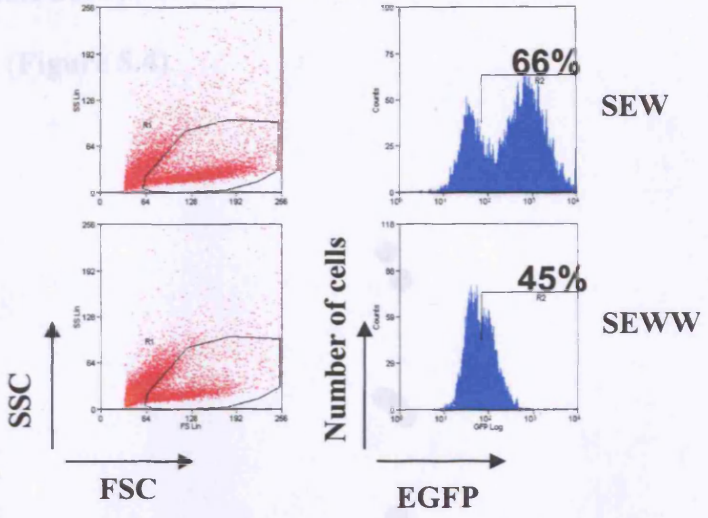


Figure 5.3 Flow cytometry plots of transduced cells
Representative plots for a) SEW and b) SEWW showing transduction levels based on EGFP expression. The EGFP levels are determined after gating for the live cells (G1) judged by the forward (FSC) and side scatter (SSC).

5.2.1.2 Bone marrow reconstitution levels

Mice were analysed after a period of 2-9 months and the levels of transduction were firstly determined by EGFP levels, measured by flow cytometry in the bone marrow. Mice reconstituted with CEW (5/5) had low levels of EGFP present up to 8 months whereas CEWW (6/6) infected cells had no EGFP positive cells in the bone marrow (**Figure 5.4**) In contrast EGFP levels in mice infected with SEW showed 14/18 mice positive for EGFP with levels of EGFP in the bone marrow ranging from 1-66% in the positive mice (**Figure 5.4**). 8/13 mice reconstituted with SEWW were positive for EGFP. The positive mice 5 months (6/6) (**Figure 5.4**) and 9 months (2/4) (data not shown) post transplant had high EGFP transduction levels in the input cells compared to 3/3 negative with low level EGFP input transduction. The levels of transduction seen in the bone marrow of SEWW mice was generally lower than that seen for SEW. In

addition the transduction levels were reduced from that observed in the infused lineage negative cells (**Figure 5.4**).

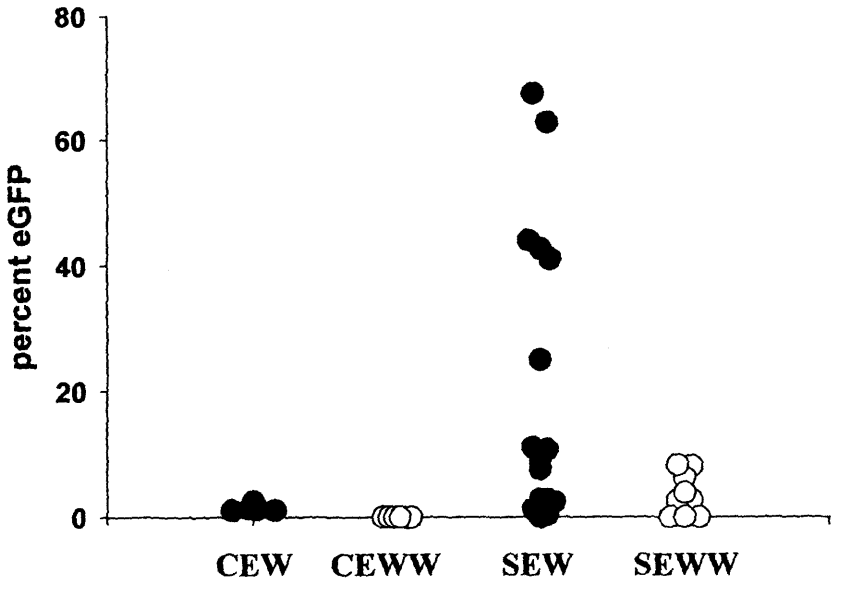


Figure 5.4 Levels of Bone marrow Transduction in Reconstituted mice
Bone marrow was removed 6 weeks to 5 months after transplantation and the level of transgene expression determined by flow cytometry for EGFP expression. CEW (n=5), CEWW (n=6), SEW (n=18), SEWW (n=9). Each point represents an individual mouse.

5.2.1.3 Secondary lymphoid organ reconstitution levels

CEW but not CEWW mice had EGFP detectable in the peripheral blood, 2-8%, spleen 2-5% and the thymus 0.1-3.4% (**Figure 5.5a**). EGFP was detectable in both SEW and SEWW transduced mice, with EGFP levels highest when SEW was used compared to SEWW. EGFP levels in peripheral blood was 42-67% and 8-18%, spleen 27-44% and 10-20% and thymus 18-35% and 0-10% for SEW and SEWW respectively (**Figure 5.5b**).

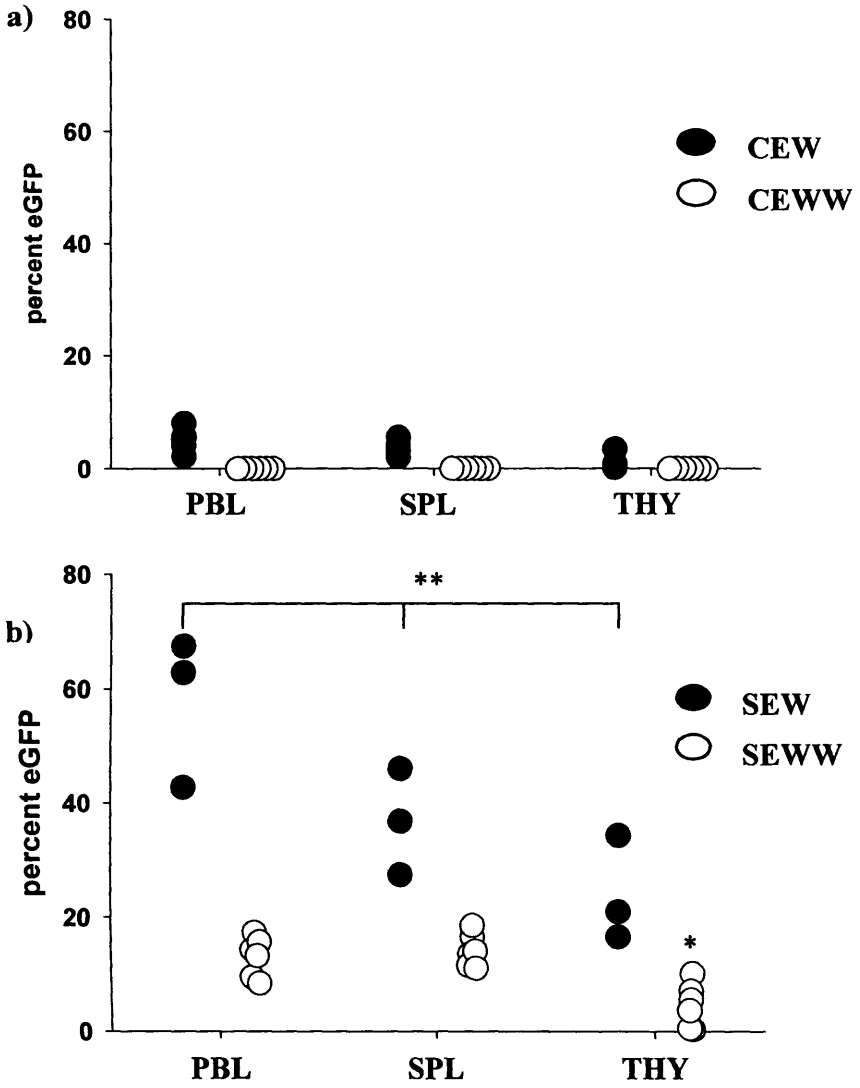


Figure 5.5 Transduction levels in secondary lymphoid tissues
3-8 months after engraftment peripheral blood (PBL), spleen (SPL) and thymus (THY) single cell suspensions from transduced mice were analysed for the presence of EGFP by flow cytometry. Each point represents a mouse. a) CEW n=5, CEWW n=6, b) SEW (n=3) SEWW (n=6). * $p< 0.005$ THY compared to PBL and SPL, ** $p<0.5$ students T test PBL compared to SPL and THY.

5.2.1.4 Lineage Analysis

In order to confirm a lineage negative multipotent murine haematopoietic stem cell had been transduced an analysis of the different lineages was performed. The presence of transduced lineage positive cells up to 5 months post infusion can only be due to transduction of multipotent cells that have engrafted and repopulated the haematopoietic system.

5.2.1.5 Peripheral Blood

Peripheral blood leukocytes from transduced mice were subdivided based upon their forward and side scatter profiles into lymphocyte (L), monocyte (M) and granulocyte (G) populations allowing the EGFP levels in each lineage to be determined (**Figure 5.6a**). The ratio of transduced lineage cells for each lineage was calculated for CEW (**Figure 5.6b**) SEW and SEWW transduced mice (**Figure 5.6c**). When the levels were compared the highest levels of transduction were seen in the myeloid lineages with the lymphoid compartment greatly reduced for all mice analysed (**Figure 5.6b+c**).

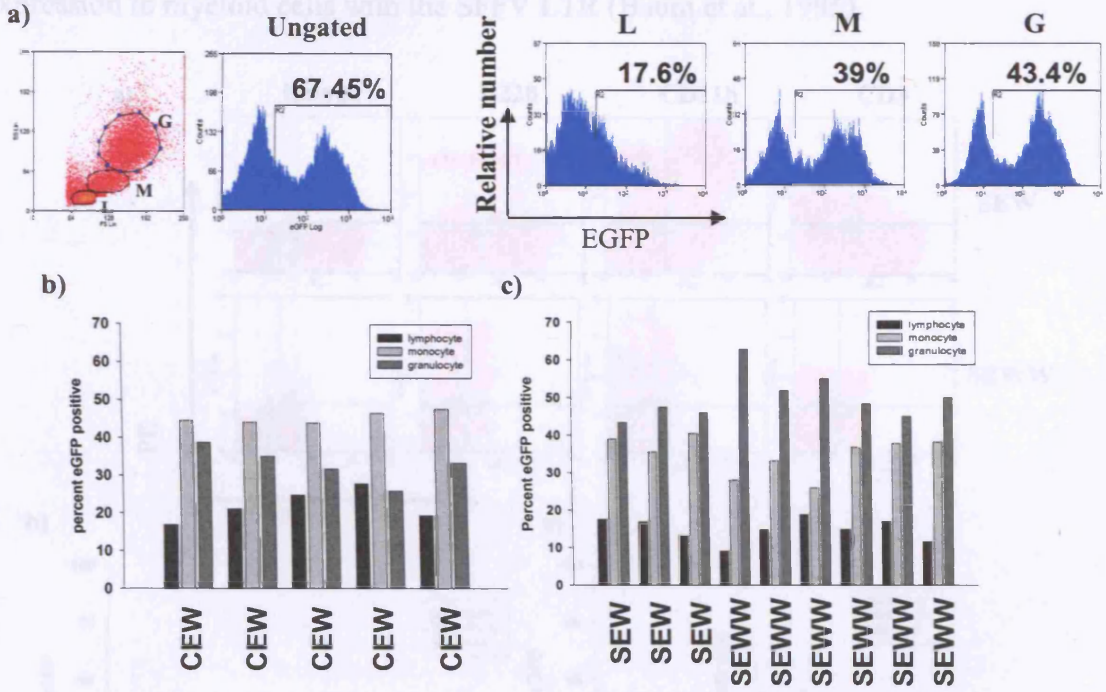


Figure 5.6 Determination of lineage transduction in peripheral blood
a) The lymphocyte (L), monocyte (M) and granulocyte (G) populations of peripheral blood from transduced mice were distinguished by their forward and side scatter in able to determine transduction levels in multiple lineages. b) + c) Bar chart showing the relative proportion of transduced cells determined for each lineage. CEW (mouse 1-5), SEW (mouse 1-3), SEWW (mouse 1-6)

5.2.1.6 Spleen

Splenocytes from CEW, SEW and SEWW transduced mice were stained with B220, CD11b and CD3 antibodies labelled with PE to distinguish B cells, myeloid cells and T

cells respectively, to determine if there was multi-lineage transduction. Flow cytometry revealed that for all transduced mice there were EGFP positive cells in all populations observed (**Figures 5.7a,b +c**). When the ratios of each lineage in the transduced population were compared the levels were evenly spread in mice transduced with CEW, with all populations transduced. In SEW and SEWW transduced mice there was generally higher EGFP expression in the myeloid lineage, and although there were often high levels of EGFP seen in B cells, the levels were lowest in the T cell compartment (**Figure 5.7c**). This corresponds well with previous data showing an increase in expression in myeloid cells with the SFFV LTR (Baum et al., 1995).

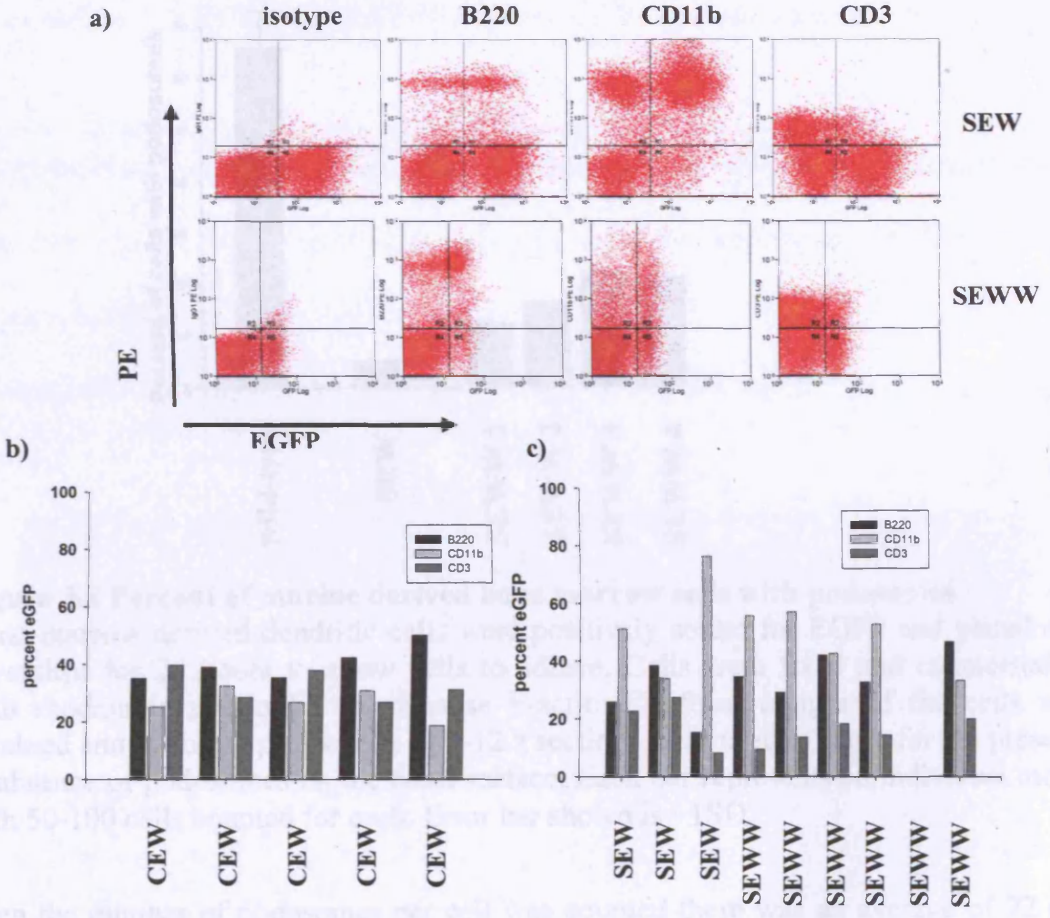


Figure 5.7 Flow cytometry of Splenocytes from Transduced Mice
a) Splenocytes from transduced mice were stained for the lineage markers B220, CD11b and CD3 labelled with PE. Representative flow cytometry plots are shown from SEW and SEWW transduced mice. b) Bar chart showing the relative proportion of transduced cells for each lineage in splenocytes determined from flow cytometry data above in CEW (mouse 1-5) and c) SEW (mouse 1-3) and SEWW (mouse 1-6) transduced mice. No data was obtainable for SEWW5 due to sample contamination.

5.2.1.7 Reconstitution of Podosomes

Bone marrow cells from reconstituted mice were differentiated into dendritic cells *in vitro*, to count podosomes. After 7 days in culture the dendritic cells were sorted on the basis of EGFP and plated onto fibronectin coated coverslips. After fixing and staining with phalloidin to reveal the F actin, confocal images were captured to enable the percent of cells with podosomes, and the number of podosomes per cell to be determined. Dendritic cells from normal mice had 65% with podosomes. Dendritic cells from SEW transduced mice had 5% with podosomes compared with 12-23% in SEWW transduced mice (**Figure 5.8**).

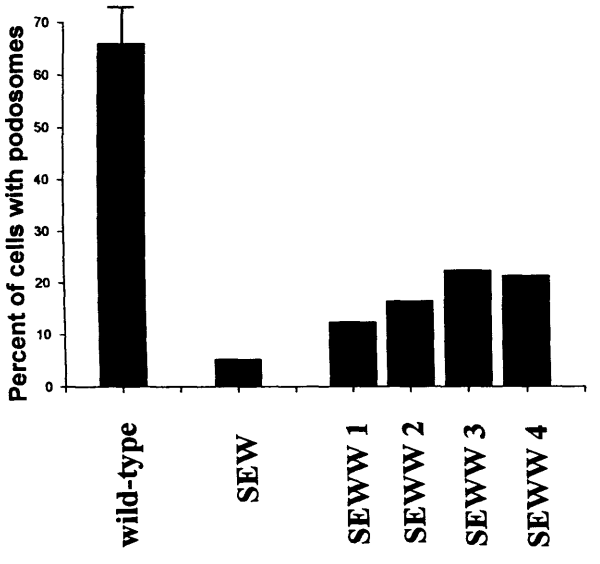


Figure 5.8 Percent of murine derived bone marrow cells with podosomes
Bone marrow derived dendritic cells were positively sorted for EGFP and plated onto coverslips for 24 hours to allow cells to adhere. Cells were fixed and counterstained with rhodamine phalloidin to visualise F-actin. Confocal images of the cells were obtained and maximal projections of 6-12 z sections were used to score for the presence or absence of podosomes on the basal surface. Each bar represents an individual mouse with 50-100 cells counted for each. Error bar shown is +1SD

When the number of podosomes per cell was counted there was an average of 22 per cell for SEWW transduced cells. This is lower than the value of 31.4 seen in dendritic cells from normal mice (**Figure 5.9**) but higher than that seen in SEW transduced and untransduced mice.

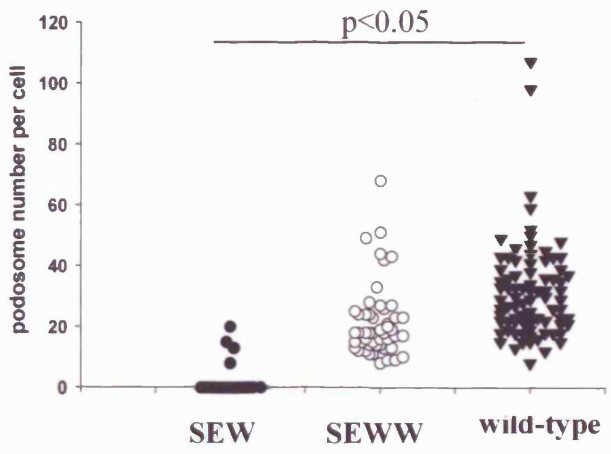


Figure 5.9 Podosomes per cell
Cells were stained with phalloidin to visualise actin and therefore podosomes. Positive cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. Values are pooled from similar treated mice in one experiment. (EGFP n=3, EGFP-WASp n=4). P value from students T test.

A representative confocal image (**Figure 5.10a-c**) of an SEWW transduced dendritic cell shows EGFP-WASp located in podosomes and co-localisation with F-actin in the podosomes (**Figure 5.10b** arrows). Further confirmation of the normal structure of the podosomes was seen by the vinculin staining in and around the podosomes (**Figure 5.10c**).

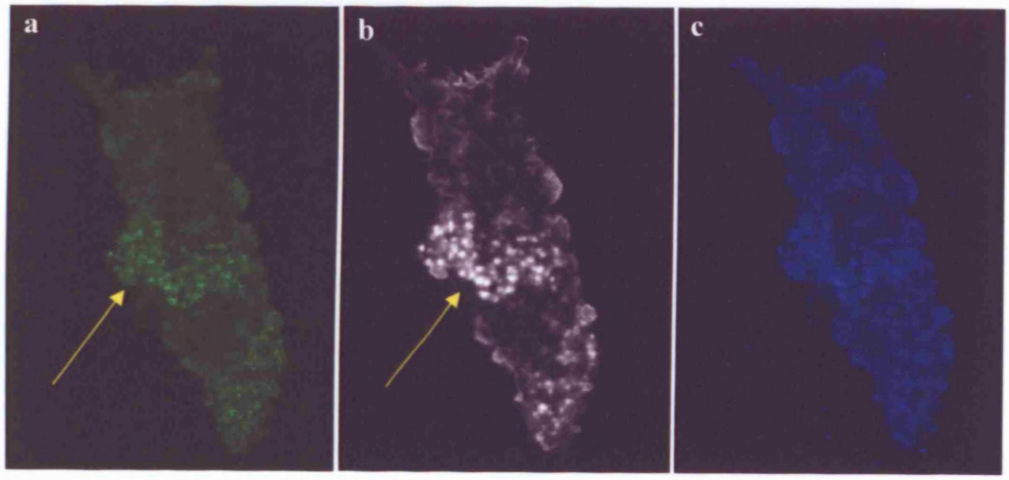


Figure 5.10 Confocal Image of Murine Dendritic Cell
Bone marrow dendritic cells from EGFP-WASp transduced mice were positively sorted on the basis of EGFP before being plated onto coverslips overnight. Cells were fixed and counterstained with rhodamine phalloidin to visualise F actin and vinculin. Arrows show the presence of podosomes. Images are maximal projections of 12 z sections.

5.2.1.8 Integrated Viral Copy Number and Human WASp Expression Levels

It is important to determine if the expression levels are dependant upon copy number and if the MOI used generates high copy numbers. In retroviral vectors each additional integration event has an increased risk of insertional mutagenesis (Li et al., 2002; Baum et al., 2003; Modlich et al., 2005), therefore high or therapeutic expression levels from few integration events is preferential.

5.2.1.8.1 Copy Number

Quantitative real time PCR was used to determine the copy number of integrated viral genomes within the bone marrow, spleen and thymus. Dilutions of the lentiviral plasmid SEWW were used to produce a standard curve against which copies of integrated genomes could be measured and quantified. In addition to be able to quantify the number of mouse genomes and therefore cells in the reaction a plasmid containing the mouse titin gene (a gift from Anne Galy, Genethon, France) was diluted to give another standard curve against which cell number could be calculated. Using this method the value for the viral genomes can be divided by the number of cells to give the copy number per cell.

In mice transduced with SEW the copy number was 5.4-8.7 for bone marrow, 5 for spleen and 1-4 for thymus (**Figure 5.11**). The decrease in copy number in thymus compared to spleen correlates well with the decreased EGFP levels, determined by flow cytometry, seen in these organs (**Figure 5.5b**). The copy number in bone marrow, spleen and thymus was 3.2-5.3, 3.5-9, and 3-3.5 in SEWW transduced mice (**Figure 5.11**) apart from one outlying value in the spleen of 95, which was probably due to

contamination. The copy number was lower than that seen in SEW transduced mice but correspondingly, the expression levels of EGFP-WASp were also lower. Unlike the values obtained from SEW transduced mice there was little corresponding reduction in copy number observed in the spleen and thymus compared to bone marrow, even though the EGFP levels were also reduced in the thymus (**Figure 5.5b**).

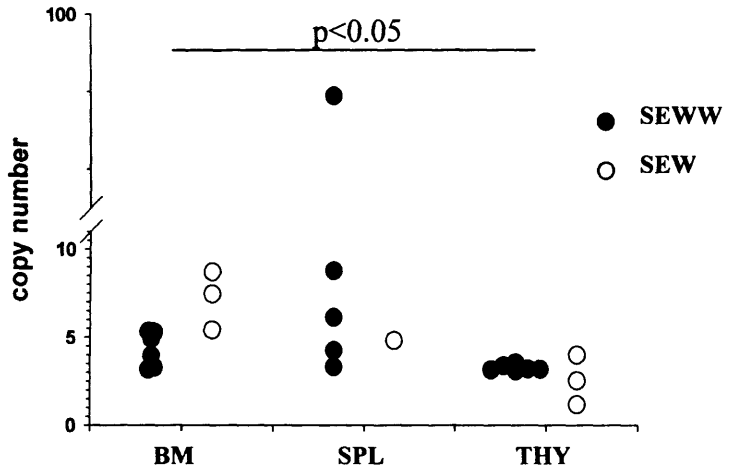


Figure 5.11 Real Time PCR on DNA to determine copy number
DNA from bone marrow (BM), spleen (SPL) and thymus (THY) from experiment 6 had the number of integrated viral genomes quantified by real time PCR. Each point represents an individual mouse taken from one experiment where the MOI was the same for SEW (n=3) and SEWW (n=6). P value is a students T test comparing values from BM to THY for both SEW and SEWW values.

5.2.1.8.2 Expression Levels

The expression level of human WASp was also calculated by quantitative real time PCR. Unfortunately there was no cell line or primary cell standard to which the transduced murine cells could be compared, as human WASp is not normally expressed in murine cells and the PCR is specific for human WASp. However the samples could be compared to each other, as they are all internally controlled using murine PGK expression. It is for this reason that all the values are compared to the levels seen from one SEWW transduced bone marrow sample (in this case mouse SEWW 1) chosen randomly.

The levels of human WASp expression were highest in the bone marrow, with some reduction in the spleen for half the samples (**Figure 5.12**). The expression levels in the thymus were lower than those observed in the bone marrow confirming the reduced EGFP expression noticed by flow cytometry (**Figure 5.12**).

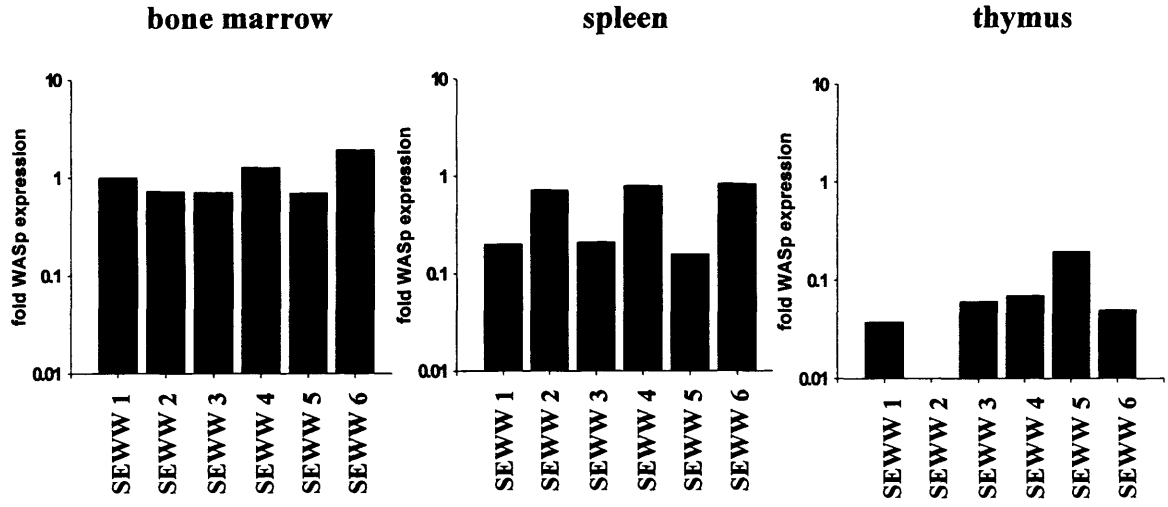


Figure 5.12 Expression of human WASp in murine bone marrow
Total RNA was collected from cell pellets from experiment 6 and the cDNA made by reverse transcription. Quantitative PCR was performed on the cDNA to determine the levels of human WASp expression compared to the ubiquitous murine protein PGK. A reference was needed and SEWW 1 was used by which all other samples were compared. Expression levels are measured on the Y axis by fold increase from the reference.

5.2.1.9 T cell Proliferation Assay

Splenic lymphocytes were incubated in the presence of anti CD3 antibody at increasing dilutions to measure the stimulatory capacity of the transduced T cells. Normal control murine T cells were able to respond strongly to all concentrations of CD3 antibody giving a high stimulation index. Mice transduced with EGFP responded weakly in comparison (**Figure 5.13**). There was stimulation in two mice transduced with EGFP-WASp at an anti CD3 antibody concentration of 1ug/ml above that observed in EGFP transduced cells. At the higher concentration of 3ug/ml 3 mice responded above that of

EGFP transduced controls (**Figure 5.13**), whereas the highest concentration of 15ug/ml showed little or no stimulation.

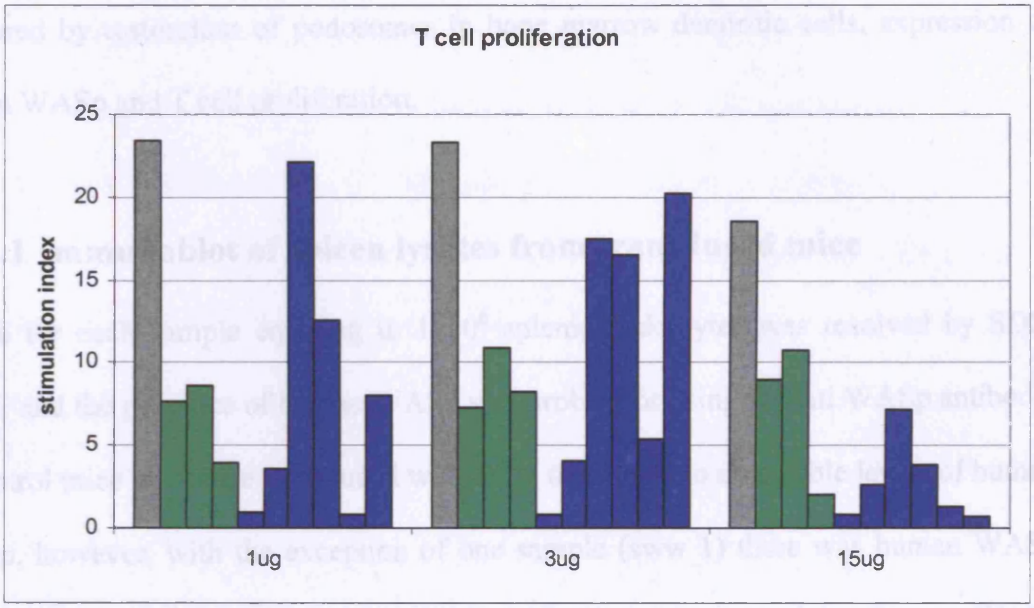


Figure 5.13 Splenic T cell proliferation in transduced mice
10^5 splenic lymphocytes from normal (■), sew (■), and seww (■) transduced mice were incubated in 96 well plates coated with anti CD3 antibody at 1ug, 3ug and 15ug/ml for 72 hours. The last 18 hours of which was in the presence of tritiated thymidine. The thymidine incorporation measured in stimulated cells compared over unstimulated cells obtained a stimulation index. sew n=3, seww n=6.

5.2.2 Reconstitution using endogenous promoter sequences

Lineage negative stem cells were harvested and transduced *ex vivo* overnight in the presence of cytokines, as previously described (**Figure 5.1**), at an MOI of 100, as determined from molecular titers, with lentiviral particles encoding for human WASp. WASp was under the transcriptional control of three different promoters, an SFFV 5' LTR, and a 500bp (Hagemann and Kwan, 1999) or 1600bp (Petrella et al., 1998) fragment of the endogenous *WAS* promoter sequence. Vectors and titers were obtained in collaboration from Anne Galy (Genethon, Evry, France). Transduced cells were

infused into lethally irradiated recipients and allowed to repopulate the haematopoietic system. Mice were culled 5 months post injection and efficiency of transduction measured by restoration of podosomes in bone marrow dendritic cells, expression of human WASp and T cell proliferation.

5.2.2.1 Immunoblot of spleen lysates from transduced mice

Lysate for each sample equating to $1x10^6$ splenic leukocytes was resolved by SDS-PAGE and the presence of human WASp was probed for using an anti WASp antibody. In control mice and those transduced with SEW there was no detectable levels of human WASp, however, with the exception of one sample (sww 1) there was human WASp detectable in lysates from all mice transduced with vectors containing human WASp (**Figure 5.14**) albeit at low levels. When the loading was checked by probing with an anti β actin antibody it was found to be similar for all samples loaded onto the gel (**Figure 5.14**).

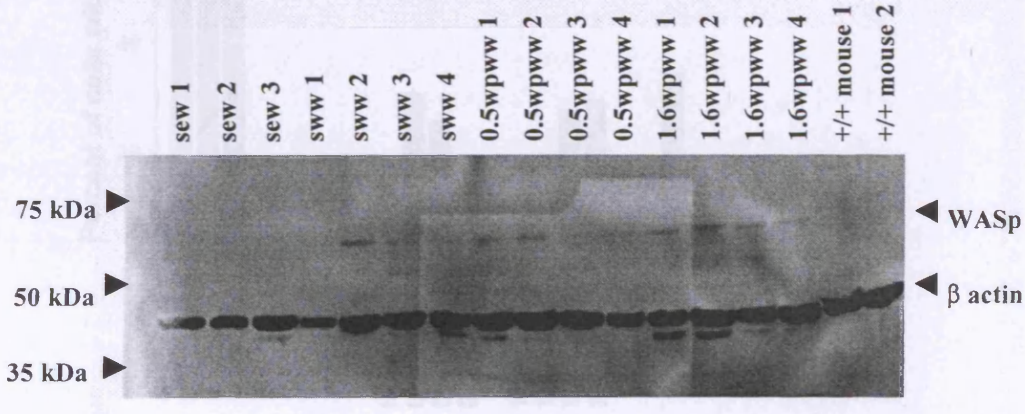


Figure 5.14 Immunoblot of splenic lysates from transduced mice
Lysates were prepared from spleens from mice transduced with WASp, EGFP and untransduced. The proteins were separated by SDS-PAGE and probed for the presence of human WASp using an anti WASp antibody. SEW = SFFV EGFP, SWW = SFFV WASp, 0.5WpWW = 500bp endogenous promoter WASp, 1.6 WpWW = 1.6 Kb endogenous promoter WASp.
The blot was also probed with an anti β actin antibody to check the loading was consistent between samples.

5.2.2.2 Podosome recovery in murine dendritic cells

Bone marrow derived dendritic cells were plated onto fibronectin coated coverslips and allowed to adhere for 24 hours. The cells were fixed and the F-actin visualised using rhodamine phalloidin. Confocal images were captured and the images used to determine the percentage of cells positive for the presence of podosomes determined. Dendritic cells from normal mice had podosomes present in 60-72% compared to 4-9% for mice transduced with SEW (**Figure 5.15**). Cells from mice transduced with WASp had podosomes present in all vectors although podosome levels could not be determined in 4 samples as the cells failed to adhere to coverslips. Cells transduced with 0.5WpWW had podosomes present in 24-30%, 1.6WpWW 26-27% and SWW 7-35% (**Figure 5.15**), which represents correction up to 50% of that observed in normal.

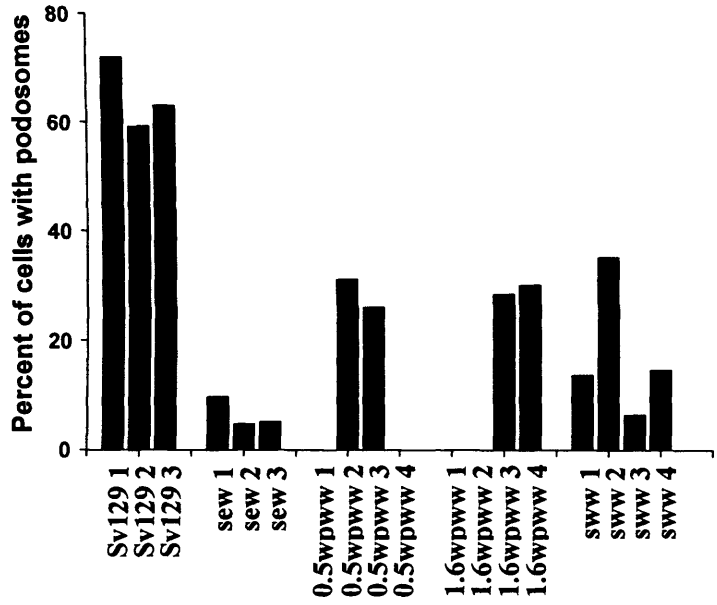


Figure 5.15 Percent of murine dendritic cells with podosomes
Murine bone marrow derived dendritic cells were plated onto fibronectin coated coverslips for 24 hours to allow cells to adhere. The cells were then fixed prior to staining with phalloidin to visualise the F actin. Confocal images were obtained and maximal projections of 8-10 sections were used to score for the presence or absence of podosomes on the basal surface. Each bar represents a mouse where 50-100 cells were counted.

To determine the quality of reconstitution the number of podosomes per cell was counted as described earlier. In cells containing podosomes the average number of podosomes per cell in normal mice was 31.9 compared to 12.9 in knockout mice. The average number per cell was 20.32, 22.62 and 30.6 for 0.5WpWW, 1.6WpWW and SWW, respectively (**Figure 5.16**). This shows that all three vectors are capable of transducing WASp knockout cells to restore podosomes to a level above that seen in knockout mice.

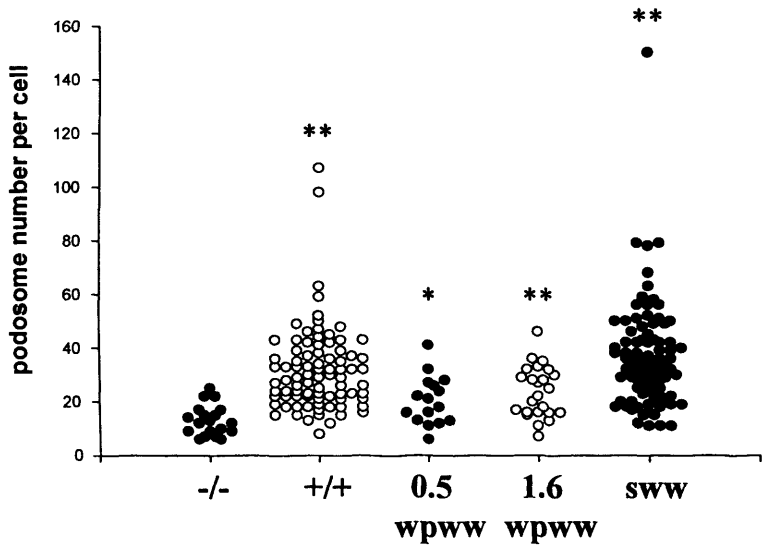


Figure 5.16 Number of podosomes per cell in dendritic cells
Cells were stained with phalloidin to visualise actin and therefore podosomes. Positive cells had the number of podosomes per cell scored and plotted. Each point represents a single cell. Values are pooled from similar treated mice in one experiment. (0.5WpWW n=2, 1.6WpWW n=2, SWW n=4). * $p<0.01$, ** $p<0.001$ students T test. Values are compared to -/-.

Representative confocal images (**Figures 5.17**) of control untransduced, SEW, SWW, 0.5WpWW and 1.6WpWW transduced dendritic cells show podosomes present (marked by arrows) in all cell populations transduced with WASp but not EGFP. No vinculin staining was performed on these samples.

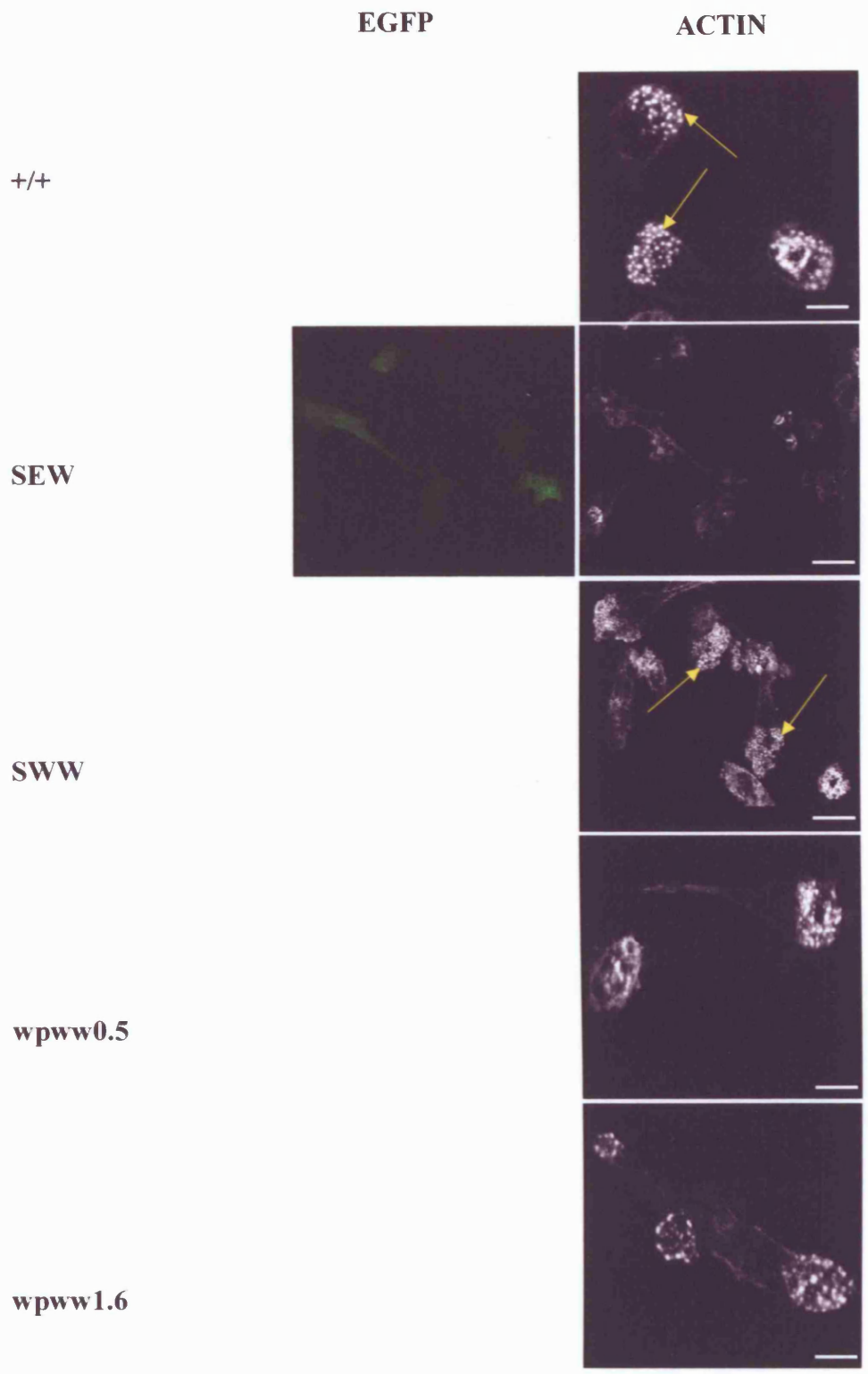


Figure 5.17 Confocal images of bone marrow dendritic cells
Bone marrow dendritic cells from normal, EGFP transduced and WASp transduced mice under the control of different promoters were plated onto fibronectin coated coverslips overnight. Cells were fixed and counterstained with rhodamine phalloidin to visualise F actin. Images shown are maximal projections of 6-12 z sections showing EGFP where present and F actin. +/+ = Sv129 control, SEW = SFFV EGFP WPRE, SWW = SFFV WASp WPRE, wpw0.5 = 500bp Wiskott promoter WASP WPRE, wpw1.6 = 1600bp Wiskott promoter WASP WPRE. Scale bar is 10um.

5.2.2.3 Integrated Viral Copy Number and Human WASp Expression Levels

As described earlier the copy number of viral genomes in the bone marrow spleen and thymus were determined by quantitative real time PCR using the same standards as previously described.

5.2.2.3.1 Copy Number

In mice transduced with SEW the copy number was 0.6-1.2 for bone marrow, 1-2.2 for spleen and 0.3-1.2 for thymus (**Figure 5.18**). The copy number for cells transduced with WASp was higher than that seen for EGFP in bone marrow, spleen and thymus. The copy number in the bone marrow was 2.6-10, 2.5-8 and 1.9-3.4 for SWW, 0.5WpWW and 1.6WpWW respectively (**Figure 5.18a**). In the spleen the copy number was 4.8-10, 6.6-10 and 3.9-5.6 for SWW, 0.5WpWW and 1.6WpWW (**Figure 5.18b**). Finally, in the thymus the copy number was 1.4-8.4, 2.2-4.2 and 2.2-4.8 for SWW, 0.5WpWW and 1.6WpWW (**Figure 5.18c**). The copy number values obtained for each organ was similar regardless of which promoter was included although the copy number was highest in the spleen. This was also seen with SEWW transduced mice (**Figure 5.11**)

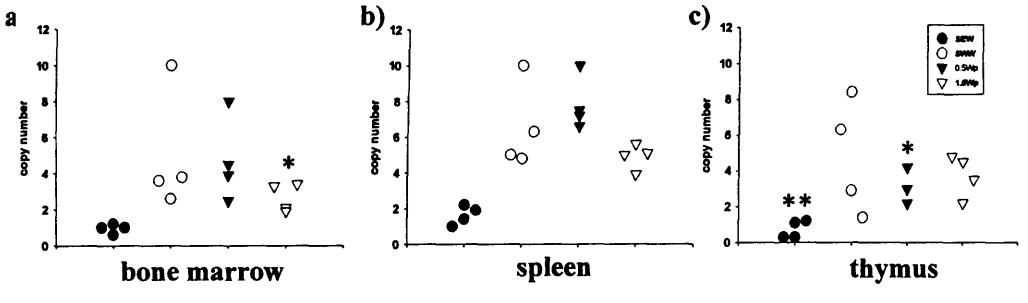


Figure 5.18 Integrated viral genome copy number
Quantitative real-time PCR was used to determine the total number of integrated viral copies in the murine genome in the bone marrow (a), spleen (b) and thymus (c). Values are corrected for the level of chimaerism based on percent Y chromosome levels. Each point represents one mouse. sew = SFFV EGFP, sww = SFFV WASp, 0.5wp = 500bp wiskott promoter WASp, 1.6wp = 1600bp wiskott promoter WASp. n=4 for all groups. *p<0.01, **p<0.05 students T test compared to values obtained in the spleen.

5.2.2.3.2 Expression Levels

The expression level of human WASp in the bone marrow and spleen was calculated by quantitative real time PCR. There was no cell line or primary cell standard to which the transduced murine cells could be compared so the bone marrow from mouse SWW1 was used as a reference to which all subsequent expression increases or decreases were obtained. In the bone marrow (**Figure 5.19**) there was some variability between samples ranging from 1-1000 fold increase in human WASp expression, whereas the spleen showed a lot less variation of 0.5-10 fold (**Figure 5.19**). The expression levels in the thymus were not determined. Use of all three promoters, lead to expression of WASp, with the average expression level highest for 0.5WpWW and lowest for 1.6WpWW.

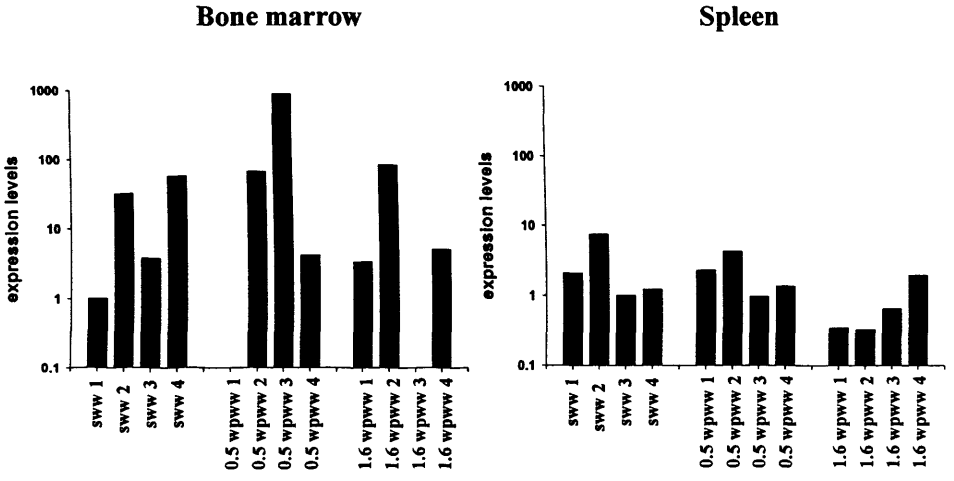


Figure 5.19 Human WASp expression levels
Quantitative PCR was performed on the cDNA reverse transcribed from the mRNA to determine the levels of human WASp expression compared to the ubiquitous murine protein, PGK. SWW1 BM was used as a reference by which all other samples were compared. Expression levels are measured on the Y axis by fold increase from the reference

5.2.2.4 T cell Proliferation Assay

Splenic lymphocytes were incubated in the presence of anti CD3 antibody at increasing dilutions to measure the stimulatory capacity of the transduced T cells. Normal control T cells were able to respond strongly to all concentrations of CD3 antibody giving a high stimulation index in 3 out of 4 mice (**Figure 5.20**). Mice transduced with EGFP responded weakly in comparison (**Figure 5.20**). There was stimulation in two mice transduced with SWW at all anti CD3 antibody concentrations above that observed in EGFP transduced cells. There was a slight increase in stimulation at the lowest CD3 dose in 1 mouse transduced with 0.5WpWW which was not seen at higher doses. No mice treated with 1.6WpWW showed a stimulation increase above SEW treated mice (**Figure 5.20**).

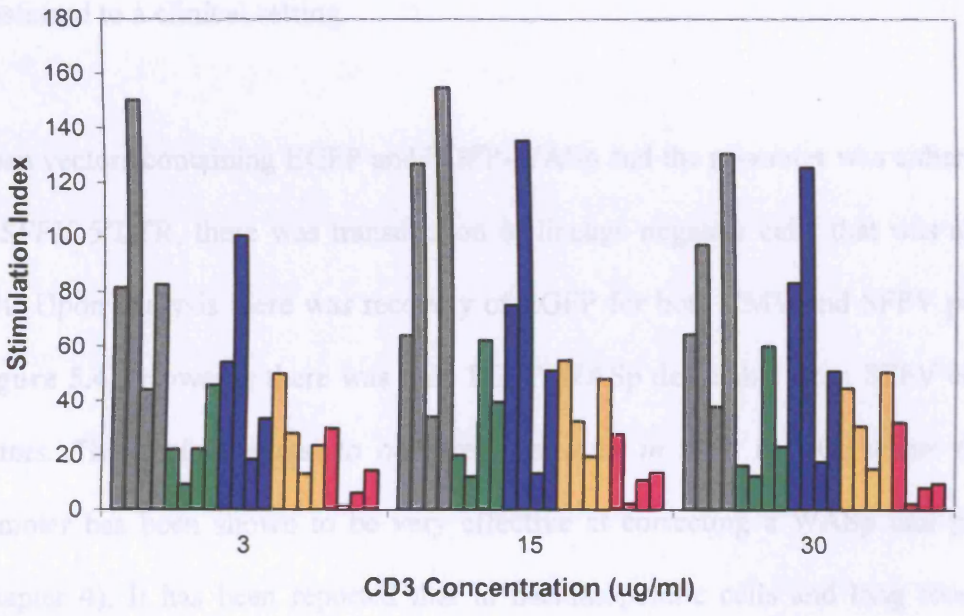


Figure 5.20 Splenic T cell proliferation in transduced mice
105 splenic lymphocytes from normal (■), sew (■), sww (■), 0.5Wp (■) and 1.6Wp (■) transduce mice were incubated in 96 well plates coated with anti CD3 antibody at 3ug, 15ug and 30ug/ml for 72 hours. The last 18 hours of which was in the presence of tritiated thymidine. The thymidine incorporation measured in stimulated cells compared over unstimulated cells obtained a stimulation index allowing comparison between mice.

5.3 Discussion

The aim of this chapter was to successfully treat a murine model of Wiskott-Aldrich syndrome by efficiently transducing lineage negative stem cells *ex vivo* with EGFP-WASp or WASp using lentiviral vectors which could then repopulate a lethally irradiated recipient. Benefits of our lentiviral system over a retroviral protocol are firstly they are self inactivating due to a U3 deletion in the 3' LTR. This should decrease the risk of insertional mutagenesis, a problem which has occurred with SCIDX1 gene therapy trials in Paris (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Additionally as an internal promoter is required this can be optimised depending upon the level of expression required (Zufferey et al., 1998). We have utilised several promoters, a CMV promoter, a viral 5' LTR and two novel endogenous WAS promoter sequences, to try to optimise a gene therapy protocol which can then be transferred to a clinical setting.

When vectors containing EGFP and EGFP-WASp and the promoter was either CMV or an SFFV 5'LTR, there was transduction of lineage negative cells that was as high as 70%. Upon analysis there was recovery of EGFP for both CMV and SFFV promoters. (**Figure 5.4**). However there was only EGFP-WASp detectable from SFFV containing vectors. This is in contrast to our previous work *in vitro* in DCs where the CMV promoter has been shown to be very effective at correcting a WASp null phenotype (Chapter 4). It has been reported that in haematopoietic cells and long term *in vivo* expression there is suppression of the CMV promoter (Liu et al., 1992; Scharfmann et al., 1991) and other promoters which has been associated with de novo methylation of the areas flanking the integration site (Jahner and Jaenisch, 1985; Challita and Kohn, 1994b). CMV mediated expression may also be dependant on cell cycle phase (An et

al., 2000) and is particularly low in T cells (Brightwell et al., 1997). For SFFV treated mice, recovery of EGFP transduced cells was similar to that seen in the input population, whereas the transduction levels in the bone marrow of EGFP-WASp transduced mice was markedly reduced. It is unclear why this should be the case as it has been reported there is a homing advantage in stem cells with WASp (Lacout et al., 2003) and somatic mosaicism has been observed in patients with spontaneous reversion mutations (Wada et al., 2001; Wada et al., 2003; Wada et al., 2004). One theory is that there is not sufficient selective pressure for T cell development in this model so a selective advantage is not seen. When cells from spleen, lymph nodes and thymus were analysed there was a reduction of EGFP and EGFP-WASp levels compared to the initial transduction levels, particularly in the thymus, supporting the lack of T cell development theory. Previous work with retroviral constructs showed lower initial transduction levels and little or no transduced cells in secondary lymphoid organs. This suggests, to gain complete reconstitution of the immune system, high transduction efficiency is required and the lentiviral vectors tested in this study may be better. In addition to multi-organ reconstitution with EGFP-WASp transduced cells, there was multi-lineage reconstitution with EGFP positive B, T and myeloid cells in the peripheral blood and spleen, which was not seen in cells transduced with EGFP-WASp using a retrovirus. Myeloid cells and B cells were the main cell types transduced with little T cell transduction. Addition of WASp has been shown to have selective advantages for T compared to B cells both in human (Konno et al., 2004) and mouse (Snapper et al., 1998), but this was not seen in this study. However higher levels of EGFP-WASp expression were generally seen in the spleen and peripheral blood compared to bone marrow which was not seen with EGFP transduced mice. Splenocytes also had a higher average copy number. Quantitative real-time PCR did not show increased expression of

WASp in spleen compared to bone marrow (**Figure 5.12**), but the system assumes that the level of house keeping gene is the same between bone marrow, spleen and thymus, which may not be the case.

Bone marrow derived dendritic cells from SFFV EGFP-WASp transduced mice had podosomes present in 12-23% of cells showing normal patterns of assembly. This is only a quarter of the level of podosomes seen in normal cells even though greater than 90% of the DCs were EGFP positive. The quality of the reconstitution was lower than that seen *in vitro* (Chapter 4) and in retrovirally transduced cells (Chapter 3) with the average number of podosomes per cell not as high as that seen in normal, although it was above that seen in untransduced cells. Although not as high as normal the levels may be sufficient for at least partial correction of the disease as it has been shown in that the mild version of Wiskott-Aldrich syndrome, XLT, there are reduced numbers of podosomes (Linder et al., 2003).

The reasons for the decreased levels of transduction after 5 months are unclear. It may be dependant on expression levels obtained initially, and data using EGFP suggests the higher the initial input transduction levels the greater the thymic reconstitution. The experimental duration may also be important but there may have been irreversible damage to the thymic microenvironment. It has been shown that the interaction of thymic epithelial cells is crucial in T cell development (Klug et al., 1998). Thymic development is abnormal in human X-linked SCID (Hale et al., 2004) and WASp has been shown to be important in T cell development (Zhang et al., 2002). Older patients submitted to a gene therapy protocol failed to engraft (Thrasher et al., 2005) compared to high levels of success with younger patients (Gaspar et al., 2004; Hacein-Bey-Abina et al., 2002; Cavazzana-Calvo et al., 2000a) suggesting time of transplant could be

crucial. This does not explain why untransduced CD3 positive cells are present in the spleen however.

A T cell proliferation assay showed that there was some lymphoid reconstitution of function. The apparent reduction in T cell reconstitution, determined by lower EGFP-WASp levels in the thymus, was shown further however where only 2 mice were able to respond to a CD3 stimulus. Strangely cells from mice that were able to respond to CD3 did not have the highest expression levels, as determined by PCR or by flow cytometry.

Further *in vivo* experiments using WASp as the transgene in place of EGFP-WASp were performed. In addition to the SFFV 5'LTR which we have shown to be successful *in vivo,* we utilised two endogenous *WAS* promoter sequences found 5' to the *WAS* start codon to see if they would be able to express WASp at sufficient levels to reconstitute then WAS phenotype. This would enable a gene therapy protocol to be developed that did not use a vector containing a constitutively active strong promoter but instead use an endogenous sequence that may provide more regulated normal expression and possibly only in haematopoietic cells (Martin et al., 2005). Lineage negative cells were transduced as previously described and infused into lethally irradiated recipients. After 5 months there was expression of human WASp, determined by western blot, in almost all samples utilising the SFFV 5'LTR, the 500bp endogenous promoter and the 1600bp endogenous promoter.

Recovery of podosomes in bone marrow dendritic cells was again used to determine the success of the reconstitution. As there was no EGFP it was not possible to select for the transgene positive populations however there were still high levels of podosomes compared to EGFP transduced that were approximately a third of that seen in normal

controls. There was a comparative level of podosome recovery in mice reconstituted with all three vectors used. Additionally the quality of the reconstitution determined by podosome number per cell was high, again with all three vectors able to achieve average podosome numbers higher than knockout or EGFP transduced controls. Although the 0.5kb and 1.6kb containing promoter WASp transduced cells had the greatest number of cells with podosomes it was the SWW transduced cells that had the most podosomes per cell.

Expression levels measured by quantitative PCR did not necessarily correlate with podosome numbers. In the bone marrow the average expression level was highest for cells transduced with 0.5WpWW, although SWW and 1.6WpWW were very similar. However the percent of cells with podosomes was increased in the cells that had high expression levels determined by real time PCR. The copy number was similar for cells transduced with all three vectors for all lymphoid tissues, with the highest levels found in the spleen. This was also seen in SEWW transduced cells, suggesting a possible maturation advantage for cells containing an integrated copy. There was no correlation between high copy number and high human WASp expression levels in the bone marrow, although there did seem to be an increase in expression with an increase in copy number in the spleen particularly for SWW and 0.5WpWW transduced cells. As mentioned, there is no standard to which the expression levels can be compared, due to human WASp being expressed in murine cells, so whether these levels are similar to that seen in normal haematopoietic cells remains to be seen.

A T cell proliferation assay in response to CD3 stimulation was performed on splenic cells and there was little stimulation above EGFP transduced cells in most of the samples tested. The mouse that did respond well was SWW 2, which did have a high

copy number and the highest expression levels determined by PCR. Furthermore the band exposed by western blot from sample SWW was visibly one of the strongest. Again the reasons for this lack of T cell reconstitution is unknown but as the mice were analysed 5 months after infusion with transduced cells it may be factors other than time constraints.

In conclusion, further to our successful reconstitution of murine WAS KO dendritic cells *in vitro*, we have successfully performed long term reconstitution of a murine WAS KO model using lentiviral vectors encoding EGFP, EGFP-WASp and WASp. This was under the transcriptional control of the retroviral SFFV 5' LTR and endogenous promoter sequences that are found upstream of the start codon of WASp. A CMV promoter containing vector was not able to reconstitute EGFP-WASp in the mouse model.

When WASp was used the reconstitution *in vivo* determined by the presence of podosomes was about a third of normal for all vectors used and the quality of reconstitution determined by podosome number was high suggesting that where there is reconstitution it will be of sufficiently high enough levels to be therapeutic.

The reconstitution with vectors containing endogenous vector sequences is encouraging as more normally regulated WASp may lead to a more normal recovery. This will allow a gene therapy trial that does not contain a viral promoter and therefore the enhancers, able to act upon other genes, found in viral LTRS. This should make the vector safer without compromising the efficacy of the treatment.

CHAPTER 6

DISCOVERY OF A NOVEL ACTIVATING MUTATION IN WAS RESULTING IN X-LINKED CONGENITAL NEUTROPAENIA

6.1 Introduction

Recently a patient with severe neutropaenia, monocytopaenia and a reversed CD4/CD8 ratio was found to have a mutation of the WAS gene that lead to a substitution in the WAS protein at L270P in the Cdc42 binding domain. Apart from the neutropaenia it was discovered that the mutation caused actin to be polymerised *in vitro* constitutively, independent of the normal regulation seen for wild type WASp (Devriendt et al., 2001). The mutation causes the amino acid lysine (L), which has a small side chain to be replaced with proline (P), which has a large side chain and is often found at helical turns. In the predicted tertiary protein structure this would prevent the basic C-terminus from folding into the pocket created by the alpha helices as this side chain is on the inner face of the α-helix. The constitutive actin polymerisation seen is thought to be due to incorrect folding of WASp preventing the inactive confirmation. This leads to a constitutively active WASp and the disease phenotype seen. This mutation could increase the further understanding of the regulation of WASp within cells and the neutropaenia and monocytopaenia associated with the unregulated expression may suggest toxicity associated with uncontrolled expression or activation of WASp.

6.1.1 Discovery of an activating mutation in WASp

A patient presented at Great Ormond Street Hospital (GOSH) with severe neutropaenia, monocytopaenia and lymphopaenia with a reversed CD4/CD8 ratio. When exons 8 and 9 (the Cdc42 binding domain) of the *WAS* gene were sequenced, a novel mutation was found. This was T36201C, which causes the amino acid substitution I294T. The mutation was checked to see if it was a naturally occurring polymorphism and was found not to be present in 100 normal individuals either European or African (the

patient is of African extraction)(P.Anclif, in press). Additionally, the mother was a carrier of the mutation and showed apparent non-random X-inactivation in T cells and neutrophils (P.Anclif, in press), which has been seen in female carriers of classical *WAS* mutations.

The mutation leads to a replacement in the Cdc42 binding domain of a neutral amino acid, isoleucine (I), with the polar amino acid threonine (T). This is thought to interfere with the folding of the C-terminus into the pocket created by the alpha helices of the Cdc42 binding domain. Additionally it is on the direct opposite side of the pocket as the previously seen mutation L270P (**Figure 6.1**) suggesting the mutation may have a similar effect and cause constitutive actin polymerisation. The aims of this chapter were to investigate the constitutively active WASp^{I294T} patient cells and the consequences of overexpression of WASp^{I294T} in cell lines to elucidate the mechanisms behind the patient phenotype.

6.2 Results

6.2.1 Characterisation of patient with WASp^{I294T}

6.2.1.1 Macrophage morphology

Peripheral blood macrophages were seeded onto glass coverslips fixed and the F-actin visualised with rhodamine phalloidin to determine any abnormalities in the actin cytoskeleton. Normal macrophages (**Figure 6.2a**) have podosomes present at a leading edge lamellopodium. Macrophages from classical WAS patients do not have podosomes and patients with less severe mutations, designated to have XLT, have reduced numbers (Linder et al., 1999; Linder et al., 2003). Macrophages from the I294T mutant had podosomes. However these podosomes were brighter than those seen in normals and

Figure 6.1 Solution structure of the autoinhibited conformation of WASp
The C terminus of WASp (orange) sits in the pocket formed by the α-helices of the GBD domain (green). The positions of the activating mutations of WASp I294T and L270P are shown in red and yellow respectively.

Image modified from: A.S.Kim, L.T.Kakalis, N.Abdul-Manan, G.A.Liu & M.K.Rosen
Ref:- MMDB Id: 13302 PDB Id: 1EJ5

were not distributed inside the cell in a normal manner. The podosomes were present in ring like structures (**Figure 6.2b**) similar to those observed in sealing zones in osteoclasts and in multiple clusters (**Figure 6.2c**). The rest of the cell had very low level of F-actin staining compared to normal and was difficult to visualise. The macrophages were stained for vinculin (**Figure 6.2d**) to determine if this was normal. There was positive staining around the actin core of the podosomes, even when in multiple clusters, showing normal assembly.

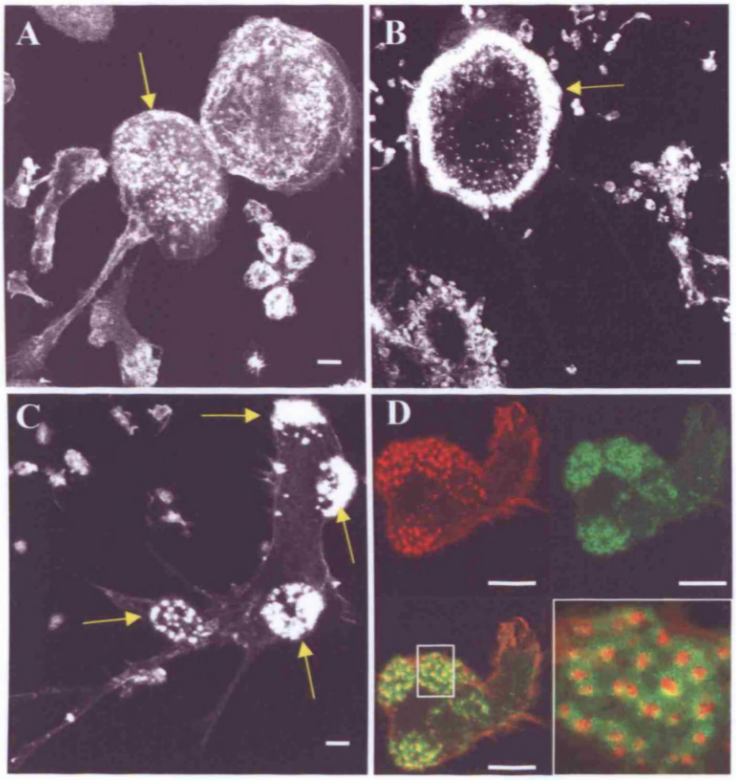


Figure 6.2 Macrophages from a patient with mutant WASp have abnormal cytoskeletal structure.
Cultured peripheral blood derived macrophages from a normal donor and ^{I294T}WASp were stained with rhodamine-phalloidin. (**A**), normal macrophages typically form clusters of podosomes behind the leading edge of the cell (arrow). (**B,C**) Patient macrophages exhibited a loss of polarisation and unusual podosome distribution, often in multiple clusters or ring structures reminiscent of osteoclast sealing zones (arrows). (**D**) Staining with anti-vinculin (FITC) antibody revealed a ring of vinculin around an actin core typical of a normal podosome structure. Scale bar is 8um.

The presence and distribution of podosomes and general cell morphology was analysed. Although when podosomes were seen they were brighter, there were actually fewer cells with podosomes in the patient than in normal (**Figure 6.3a**) and there were more cells with multiple clusters of podosomes (**Figure 6.3b**) than normal. In the patient almost 10% of the cells had podosomes in a ring structure (**Figure 6.3c**), which was hardly ever seen in normal. Podosomes are dynamic structures that are rapidly assembled and disassembled in response to various stimuli (Linder et al., 1999). Using interference reflective microscopy podosome dynamics were assessed. The turnover of podosomes in I294T macrophages was increased compared to normal (P Ancliff, in press) suggesting abnormal actin regulation.

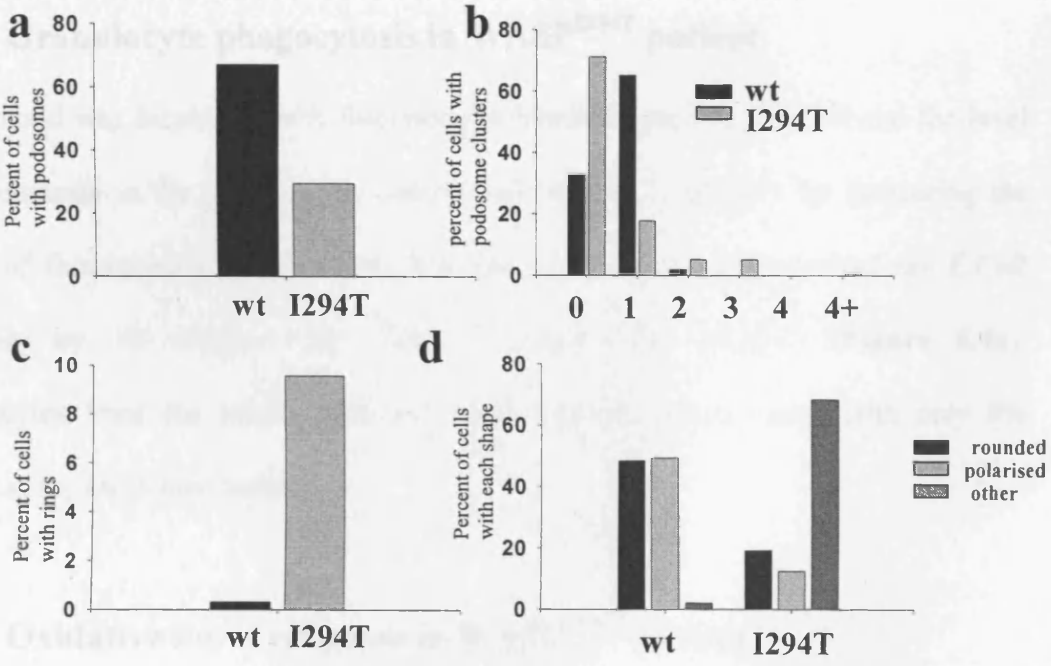


Figure 6.3 Quantification of cytoskeletal structures in patient macrophages
(**a**) The percent of cells with podosomes (**b**) the number of clusters per cell and (**c**) the percent with osteoclast like sealing zones was quantified from confocal images of macrophages stained with rhodamine phalloidin. (**d**) Cell shape was assessed on 3 characteristics of rounded, polarised and other (where large multiple processes were observed see **Figure 2b+c**).

Next, the cell shape was characterised into 3 categories (**Figure 6.3d**). Normal macrophages were either rounded, or polarised with a definite leading edge. In contrast although some patient macrophages were polarised or rounded, the predominant shape was an abnormal one with large and frequent protrusions (**Figure 6.2b and 6.2c**). This abnormal macrophage spreading, elongation and dispersion was further quantified using delineated images captured in Adobe Photoshop ® using a Mathematica notebook. I294T macrophages showed less elongation, more spreading and increased dispersion due to the membrane protusions, compared to normal control (P Ancliff, in press). This is in contrast to WASp null patient macrophages, which have increased elongation and reduced dispersion.

6.2.1.2 Granulocyte phagocytosis in WASP^{I294T} patient

Whole blood was incubated with fluorescently labelled opsonised *E.Coli* and the level of phagocytosis in the granulocytes determined by flow cytometry by measuring the percent of fluorescently positive cells. Normal granulocytes phagocytosed the *E.Coli* efficiently by 10 minutes with 90% of granulocytes positive (**Figure 6.4a**). Granulocytes from the patient had very much reduced phagocytosis with only 6% positive at the same time point.

6.2.1.3 Oxidative burst response in WASP^{I294T} patient

Whole blood was collected and the oxidative burst of neutrophils in response to different stimuli was measured. The oxidative burst was measured by the oxidation of non-fluorescent dihydroxyrhodamine (DHR) to fluorescent rhodamine by free radicals released by the cell. PMA was used to check responsiveness and to obtain a maximal response. Both control and patient cells were able to generate an oxidative burst in

response to PMA (**Figure 6.4b**). However when the physiological stimuli *E.Coli* and f-met-leu-phe (fMLP) were used there was a clear difference between normal and patient with the patient cells unable to generate a response. This shows that although the patient cells can generate an oxidative burst, they are unlikely to due to an inability to respond to a physiological stimulus.

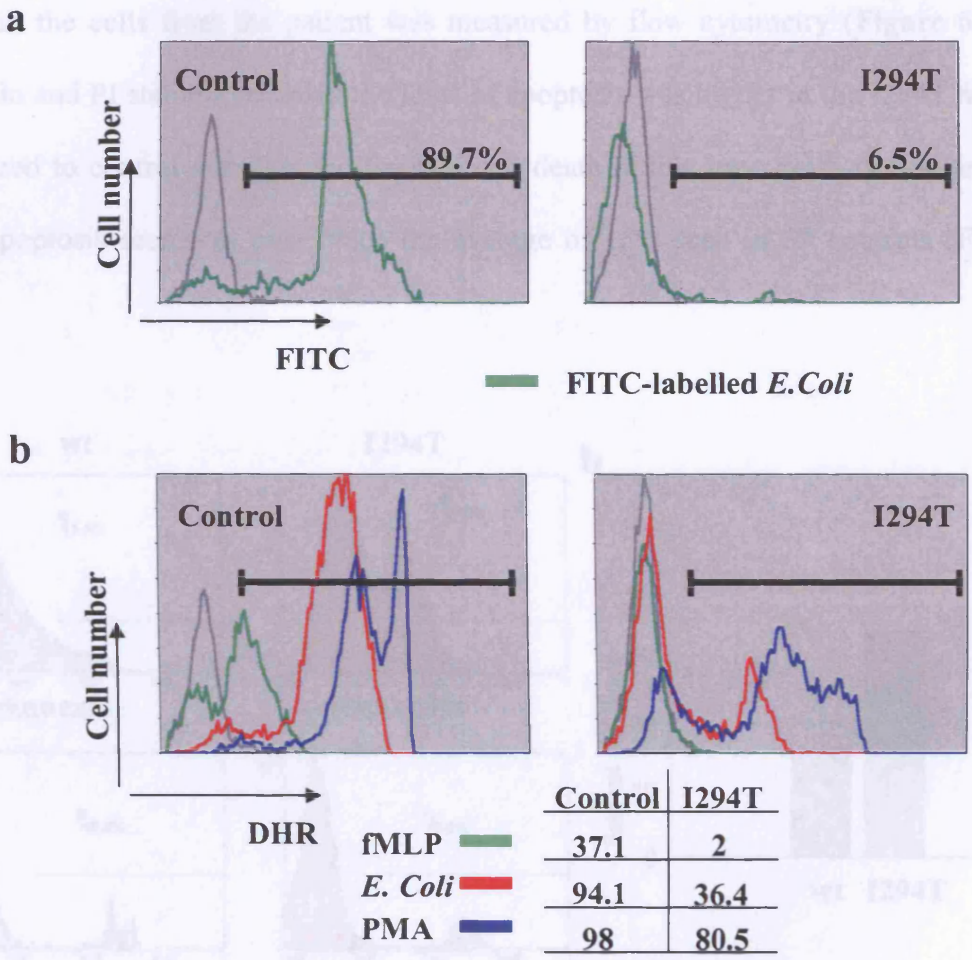


Figure 6.4 Phagocytosis and oxidative burst defects in patient neutrophils (**a**)Whole blood incubated with opsonised *E.Coli* for 10 minutes at 37°C revealed a defect in phagocytosis compared to normals. (**b**) Although oxidative burst responses, measured by rhodamine release from DHR, could be seen with PMA, the physiological stimuli of fMLP and *E.Coli* failed to elicit as strong a response in patient cells.

6.2.1.4 Viability of haematopoietic cells

6.2.1.4.1 CH-11 mediated apoptosis levels in peripheral blood

The mononuclear cell fraction of Ficol separated whole blood was incubated for 48 hours in the presence of IL-2 and PHA before induction of FAS restricted apoptosis using the anti CD95 antibody CH-11. The level of apoptosis and cell death after 48 hours in the cells from the patient was measured by flow cytometry (**Figure 6.5a**). Annexin and PI staining revealed the level of apoptosis was higher in the I294T mutant compared to control although the level of cell death at this time point is similar. The 33% apoptosis seen was over twice the average of 15% seen in 33 controls (**Figure 6.5b**).

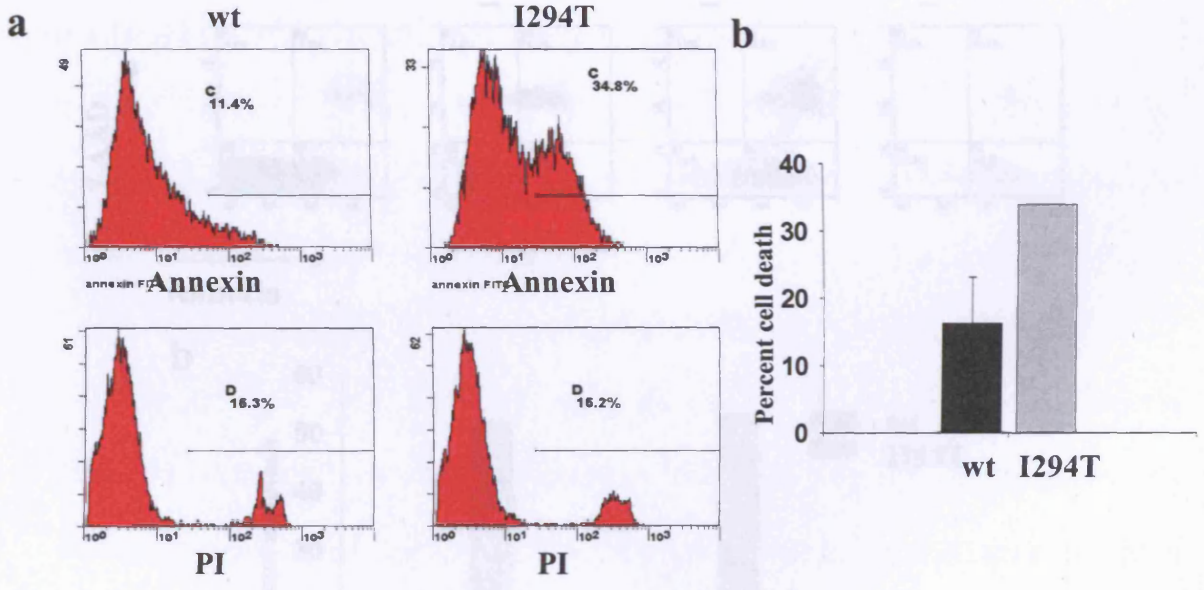


Figure 6.5 Increased levels of apoptosis in cultured lymphocytes
(**a**) Ficolled peripheral blood was incubated with CH-11, an anti CD95 antibody and the levels of apoptosis determined by annexin V and PI staining. (**b**) There was an increase in apoptosis compared to those obtained from 33 control individuals (range 5.8-36.2 with SD 6.8)

6.2.1.4.2 Spontaneous apoptosis levels in whole bone marrow

Spontaneous apoptosis in freshly derived bone marrow was measured by staining with Annexin and cell surface markers before measuring levels of fluorescence within 15 minutes. The cells were gated on their surface markers (**Figure 6.6a**) and the apoptosis measured. When compared to normal (**Figure 6.6b**) the levels of apoptosis were approximately 4-fold higher in CD34+ (14% and 52%), CD15+ (5% and 15%) and CD33+ (15% and 53%). These data suggest an increased propensity for apoptosis, which is particularly noticeable in precursor and myeloid cells, and may be the underlying reason for the neutropaenia and monocytopaenia.

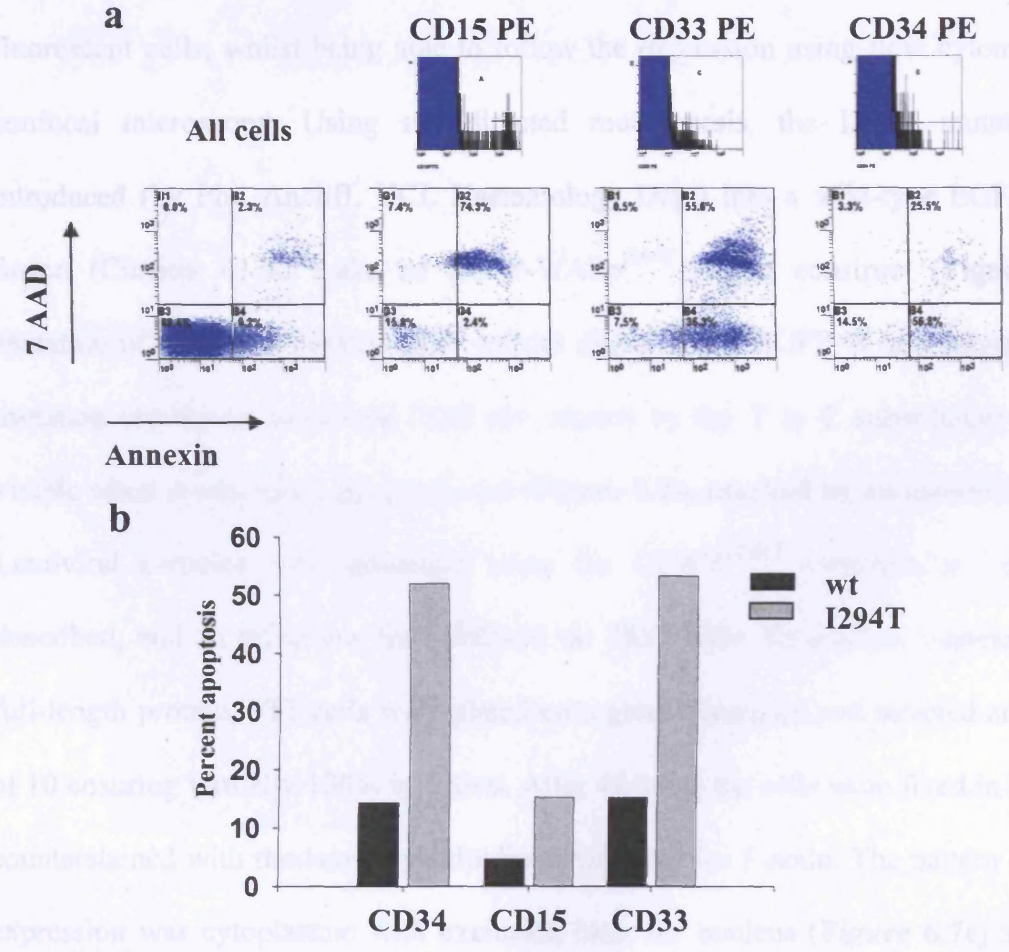


Figure 6.6 Spontaneous apoptosis in fresh bone marrow is increased in patient cells

(**a**) Whole bone marrow was collected and stained for the lineage markersCD15, CD33 and CD34, annexin and 7AAD to determine apoptosis levels within bone marrow lineages. (**b**) Apoptosis is increased, particularly in the more primitive bone marrow precursor populations.

6.2.2 Generation of a lentivirus encoding EGFP-WASp^{I294T}

In order to further characterise the I294T mutation and the effect of this mutation in cell culture with regards to toxicity of WASp expression, a HIV-1 based lentiviral vector was constructed that encoded for the mutant protein as a fusion with EGFP. We have previously shown an ability to express a fusion of EGFP and WASp in different cell types after infection with a lentiviral vector and for the fusion to act normally in human macrophages and murine dendritic cells (Chapter 4). Transduction with a lentivirus encoding EGFP-WASp allows some degree of regulated expression, by incorporating different promoters with varying strengths, varying the MOI and selection of EGFP low fluorescent cells, whilst being able to follow the expression using flow cytometry and confocal microscopy. Using site directed mutagenesis, the I294T mutation was introduced (by Phil Ancliff, UCL Haematology Dept) into a wild-type EGFP-WASp fusion (Chapter 4) to make an EGFP-WASp^{I294T} fusion construct (**Figure 6.7a**). Mutation of *WAS* was confirmed by a DdeI digest of the EGFP-WASp fragment. The mutation created an additional DdeI site created by the T to C substitution and was visible when separated on an agarose gel (**Figure 6.7b, marked by an asterisk**).

Lentiviral particles were generated using the CEWW^{I294T} construct, as previously described, and an infectious titer obtained on 293T cells. To confirm expression of a full-length protein, 3T3 cells were plated onto glass coverslips and infected at an MOI of 10 ensuring virtually 100% infection. After 48 hours the cells were fixed in PFA and counterstained with rhodamine phalloidin to visualise the F-actin. The pattern of EGFP expression was cytoplasmic with exclusion from the nucleus (**Figure 6.7c**) and there was considerable co-localisation with actin mimicking the pattern previously seen with wild-type EGFP-WASp suggesting normal protein expression.

An immunoblot was performed on cell lysates from infected 293T cells and probed for WASp. Cells infected with CEW did not have WASp (**Figure 6.7d**). However cells infected with either CEWW^{WT} or CEWW^{I294T} had protein that was consistent in size with a fusion of EGFP and WASp. There was a slight mobility shift in EGFP-WASp^{I294T}, which has been seen in westerns from patient samples (personal communication, P. Ancliff), suggesting the presence of EGFP had no deleterious effect.

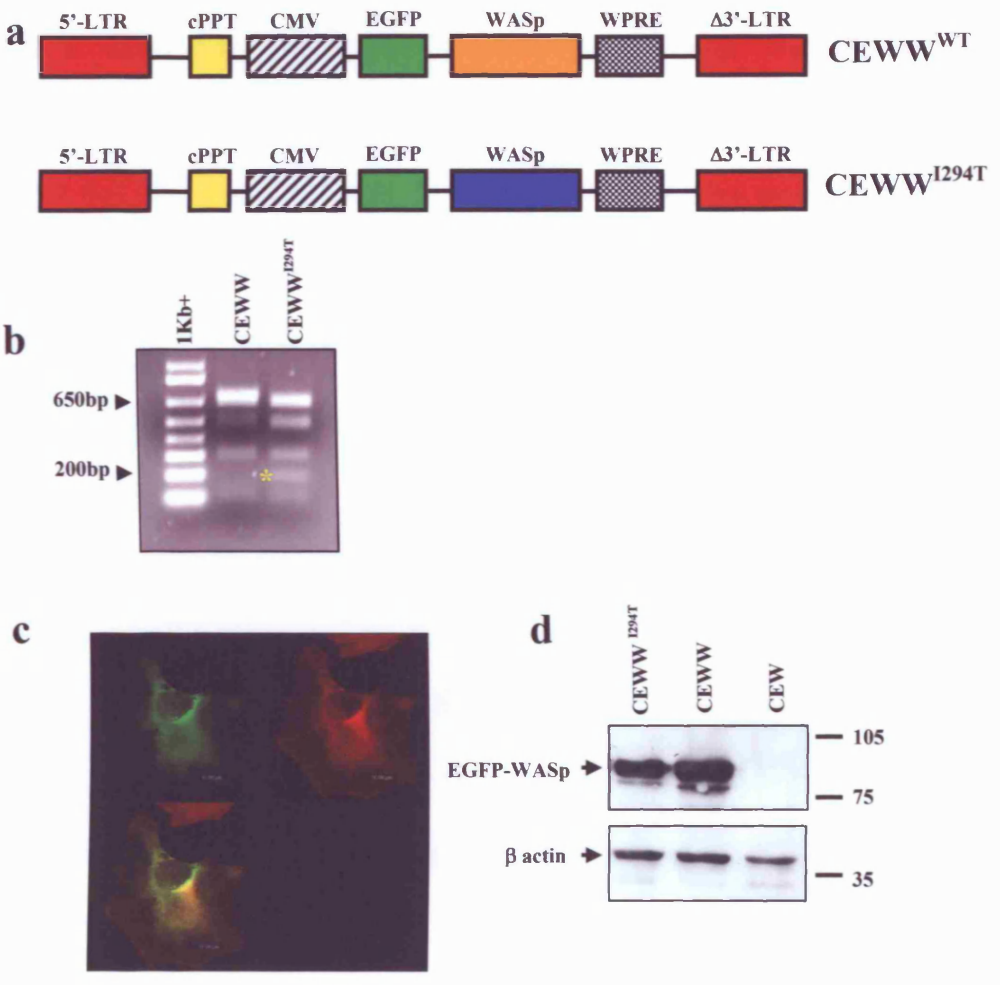


Figure 6.7 Creation of a lentiviral vector containing EGFP-WASp^{I294T}
(**a**) The I294T mutation was created by insertional mutagenesis using PCR with primers containing the mutation in the lentiviral vector. (**b**) Creation of the mutation was confirmed by digestion of the EGFP-WASp fragment with Dde-I revealing an extra band at 200bp (*) when run on a 1% agarose gel. (**c**) Infection of 293T cells with virus containing EGFP-WASp^{I294T} (green) lead to an expression pattern similar to that previously seen for EGFP-WASp^{WT} with predominantly cytoplasmic expression and co-localisation with F-actin (red). (**d**) An immunoblot probed with anti WASp antibodies confirmed a 90kDa protein was being made, although a subtle mobility shift was noticeable in EGFP-WASp^{I294T} lysates.

6.2.2.1 Infection of Bac1 macrophages with lentiviral vectors

Bac1 macrophages are an M-CSF dependant murine macrophage cell line derived from splenic adherent cells transformed with SV40 DNA mutated in the origin of replication (Schwarzbaum et al., 1984). As they are a haematopoietic cell line, WASp activity, and therefore mutant WASp activity, may be revealed in a more physiological manner. Bac1 cells maintained in culture with 10ng/ml M-CSF were plated on coverslips and transduced at an MOI of 40 and incubated for 72 hours to allow maximal expression. After 72 hours there was greater than 95% transduction efficiency with CEW and CEWW^{I294T} (**Figure 6.8a,b**). The gross morphology revealed with F-actin staining showed that Bac-1 macrophages do not normally assemble podosomes (**Figure 6.8c**). However when they were transduced with vectors containing EGFPWASp^{I294T} podosomes were formed (**Figure 6.8d,e,f**). Apart from those seen at a leading edge, podosomes were often in multiple clusters and occasionally in ring like formations (**Figure 6.8g,h,i**) similar to that observed in patient macrophages. EGFPWASp^{I294T} localised to the podosome structures (**Figure 6.8d,e**) and their nature confirmed by their localisation to the lower surface of the cell and a characteristic ring of vinculin around the actin core (**Figure 6.8e,f**). The frequency of podosome assembly was quantified by counting random fields of 100-150 cells. Overall 9% of CEWW^{I294T} transduced Bac-1 macrophages had podosomes compared to just 1% of CEWW^{WT} (**Figure 6.8j**). This ability to generate podosomes is indicative of an ability to stimulate actin polymerisation. This was confirmed in an *in vitro* actin polymerisation assay where WASp coated Sepharose beads were able to polymerise actin when incubated in cell extracts from U937 cells. This could be blocked with cytochalasin D confirming it is newly formed F-actin and not monomeric G-actin (P.Ancliff in press) formed by WASp^{I294T}.

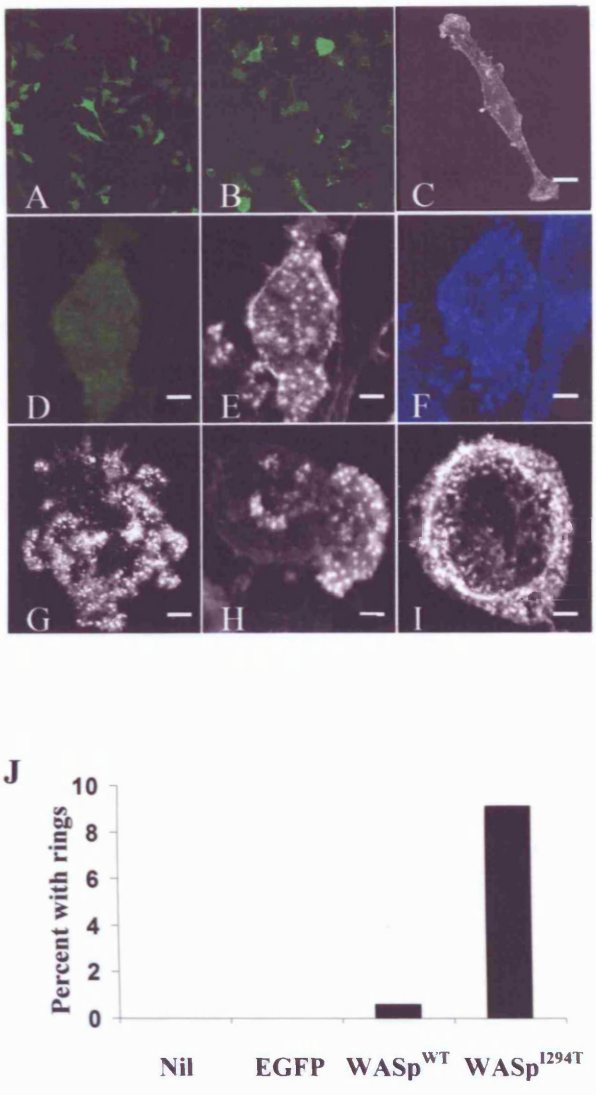


Figure 6.8. EGFP-WASp^{I294T} induces the formation of podosomes in Bac-1 macrophages.
A, B Efficiency of transduction observed by EGFP expression was greater than 95% for each well, as shown for vectors encoding EGFP (**A**) and EGFP-WASp^{I294T} (**B**). **C**. Bac-1 macrophages do not normally assemble podosomes (stained with rhodamine phalloidin). **D, E & F**. Cells transduced with vectors encoding EGFP-WASp^{I294T} assemble podosomes, in which EGFP (D) localises to the podosome actin (stained with rhodamine phalloidin) core (**E**). The nature of podosomes was confirmed by a characteristic ring of vinculin (anti-vinculin Cy5) surrounding the actin core (**F**). **G, H & I**. The distribution of podosomes mimics that of primary cells from WASp^{I294T} mutant cells, forming multiple clusters and ring formations (stained with rhodamine phalloidin). **J**. Percentage of transduced macrophages with one or more podosomes according to mutant inserted.

6.2.2.2 Viability of transduced HT1080 cells is diminished

Over a 15-day period the EGFP levels, DNA levels and cell viability were measured in transduced HT1080 cells. Cells infected with CEW or CEWW^{WT} maintained their EGFP levels (**Figure 6.9**) although cells infected with CEWW^{WT} exhibited some variation in the transduction levels over time. In contrast cells infected with CEWW^{I294T} reduced significantly in number from 31% to 7% at an MOI of 1 and from 90% to 38% at an MOI of 10, being outgrown by untransfected cells at both MOI's tested (**Figure 6.9**).

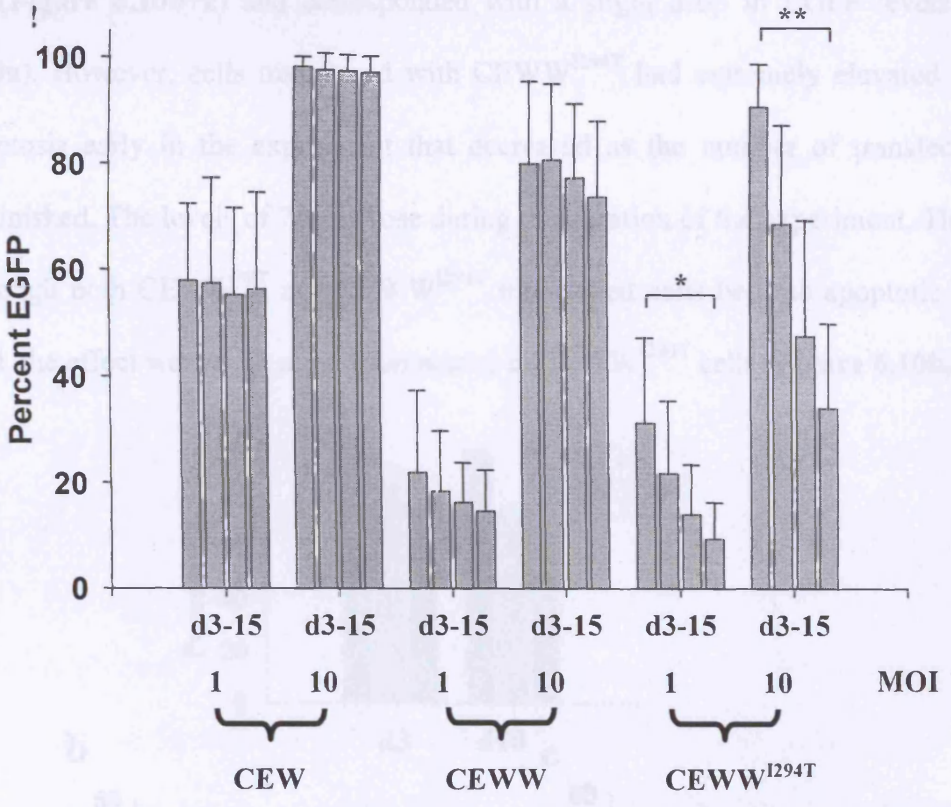


Figure 6.9 Infection of HT1080 cells leads to inhibited growth of EGFP-WASp^{I294T} expressing cells

HT1080 cells were transduced with lentiviral vectors encoding EGFP (CEW), EGFP-WASp^{WT} (CEWW) and EGFPWASp^{I294T} (CEWW^{I294T}), at a multiplicity of infection of 1 and 10. Percentage of EGFP positive cells were scored at 3,6,10,15 days (represented by vertical bars) after transduction by flow cytometry, and were stable throughout the observation period for EGFP and EGFP-WASp^{WT} with no observable toxicity. In contrast, cells transduced with EGFP-WASp^{I294T} were steadily outgrown by non-transduced cells, indicating an inhibitory effect on cell growth even at low copy number. Bars represent the average of 6-8 experiments with standard deviation. T-Test *p = 0.018, **p = 0.00001.

The viability of the HT1080 cells was measured by staining with Annexin to determine apoptosis levels and 7AAD to measure total cell death. Once again high levels of transduction was seen 3 days post infection, which diminished over time by 15% for CEWW^{WT} and by over 30% for CEWW^{I294T} (**Figure 6.10a**). Cells transduced with CEW did not have high levels of apoptosis either at the beginning of the culture period or at the conclusion (**Figure 6.10b**), which was mirrored by a low level of cell death throughout the experiment (**Figure 6.10c**). Cells transduced with CEWW^{WT} had elevated levels of apoptosis early after infection followed by increased cell death by day 10 (**Figure 6.10b+c**) and corresponded with a slight drop in EGFP levels (**Figure 6.10a**). However, cells transduced with CEWW^{I294T} had extremely elevated levels of apoptosis early in the experiment that decreased as the number of transfected cells diminished. The levels of 7AAD rose during the duration of the experiment. This shows although both CEWW^{WT} and CEWW^{I294T} transfected cells became apoptotic and then died, the effect was much more pronounced in CEWW^{I294T} cells (**Figure 6.10b,c**).

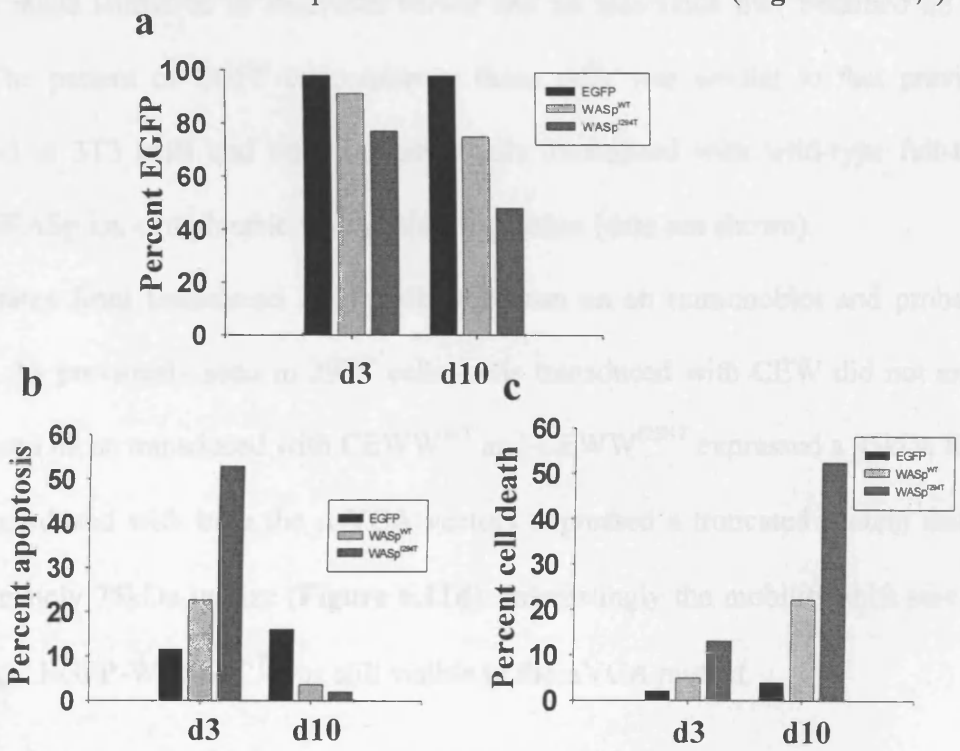


Figure 6.10 Apoptosis levels in transduced HT1080 are increased
HT1080 cells infected with EGFP, EGFP-WASp^{WT} and EGFP-WASp^{I294T} were harvested from culture and counterstained with Annexin V PE and 7AAD to determine apoptosis and cell death levels. (**a**) EGFP levels were monitored and Annexin (**b**) and 7AAD (**c**) levels determined from the EGFP positive populations using flow cytometry.

6.2.3 Cloning of VCA domain deleted lentiviral constructs

The "verprolin cofilin acidic" or VCA domain at the c-terminus of WASp is where monomeric actin and ARP2/3 bind, leading to initiation of new F-actin in response to stimuli (Higgs et al., 1999; Symons et al., 1996). In order to confirm whether the cellular defects seen and death of transfected cells were due to unregulated activation of the actin cytoskeleton, VCA deletion mutants of wild-type WASp and ^{I294T}WASp were constructed. The CEWW^{WT} and CEWW^{I294T} vectors were digested with EcoRI, the relevant fragments purified from an agarose gel and re-ligated, thus removing a 231bp fragment, whilst keeping the N-terminal EGFP in frame to maintain the fusion. This corresponds to the terminal 77 amino acids, which make up the VCA domain (shown in **Figure 6.11a**) and resulted in viral vectors that encoded a truncated version of EGFP-WASp (**Figure 6.11b**). The cloning was confirmed by digestion with EcoRI (**Figure 6.11c**) revealing the missing band on an agarose gel. These Δ-VCA mutant vectors were used to make lentivirus as described earlier and an infectious titer obtained on 293T cells. The pattern of EGFP expression in those cells was similar to that previously observed in 3T3 cells and other adherent cells transduced with wild-type full-length EGFP-WASp i.e. cytoplasmic with nuclear exclusion (data not shown).

Cell lysates from transduced 293T cells were run on an immunoblot and probed for WASp. As previously seen in 293T cells, cells transduced with CEW did not express WASp and those transduced with CEWW^{WT} and CEWW^{I294T} expressed a 95kDa fusion. Cells transduced with both the Δ-VCA vectors expressed a truncated protein that was approximately 75kDa in size (**Figure 6.11d**). Interestingly the mobility shift seen with full length EGFP-WASp^{I294T} was still visible in the ΔVCA mutant.

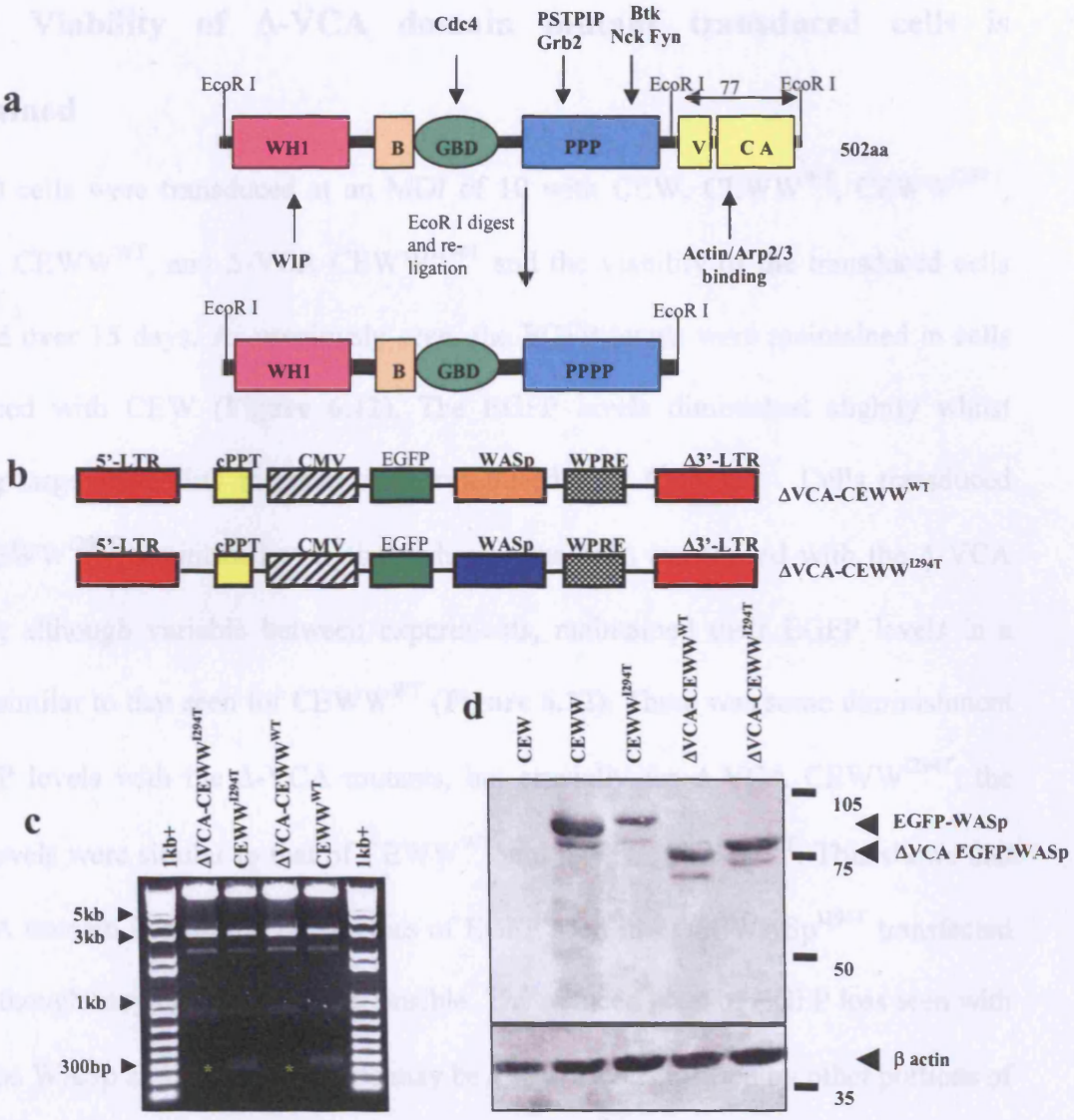


Figure 6.11 Creation of VCA deleted mutants of EGFP-WASp lentiviral constructs

(**a**) Digestion of EGFP-WASp^{WT} and EGFP-WASp^{I294T} with EcoRI allowed the deletion of the actin binding C-terminal VCA domain thus creating lentiviral constructs without the actin-binding domain. (**b**) Cartoon depiction of constructs created. (**c**) Digestion with EcoRI shows the loss of a 200bp band (*) which is the VCA domain. (**d**) Immuno-blot from lysates of HT1080 cells infected with EGFP, EGFP-WASp, EGFP-WASp^{I294T}, Δ-VCA EGFP-WASp and Δ-VCA EGFP-WASp^{I294T} probed with an anti WASp antibody. A smaller band corresponding to the deletion mutants was seen. Interestingly the mobility shift was seen for both full length and deletion mutants. Comparable loading was checked by probing for β-actin.

6.2.3.1 Viability of Δ-VCA domain mutant transduced cells is maintained

HT1080 cells were transduced at an MOI of 10 with CEW, CEWW^{WT}, CEWW^{I294T}, Δ–VCA CEWW^{WT}, and Δ-VCA CEWW^{I294T} and the viability of the transduced cells followed over 15 days. As previously seen, the EGFP levels were maintained in cells transduced with CEW (**Figure 6.12**). The EGFP levels diminished slightly whilst showing large variability in those cells transduced with CEWW^{WT}. Cells transduced with CEWW^{I294T} diminished over time whereas the cells transduced with the Δ-VCA mutants, although variable between experiments, maintained their EGFP levels in a pattern similar to that seen for CEWW^{WT} (**Figure 6.12**). There was some diminishment of EGFP levels with the Δ-VCA mutants, but crucially for Δ-VCA CEWW^{I294T}, the EGFP levels were similar to that of CEWW^{WT} and Δ-VCA CEWW^{WT}. This shows that the VCA domain is required for the loss of EGFP seen in EGFPWASp^{I294T} transfected cells, although may not be solely responsible. The reduced level of EGFP loss seen with wild-type WASp and Δ-VCA mutants may be due to effects exerted by other portions of the WASp protein, or due to the fusion with EGFP.

The viability of the Δ-VCA vector transduced HT1080 cells compared to full length vectors was measured over the 15 day period, using apoptosis and cellular death markers as described previously. At day 3 levels of apoptosis and cell death were comparable in cells transduced with all vectors (**Figure 6.13b+c**). By day 10 however, a time point where the EGFP population was diminishing in the CEWW^{I294T} population with a corresponding increase in cell death, the levels of apoptosis and cell death in Δ-VCA mutants was comparable or lower than CEWW^{WT} showing the cells were mainly healthy and maintaining their viability (**Figure 6.13b+c**).

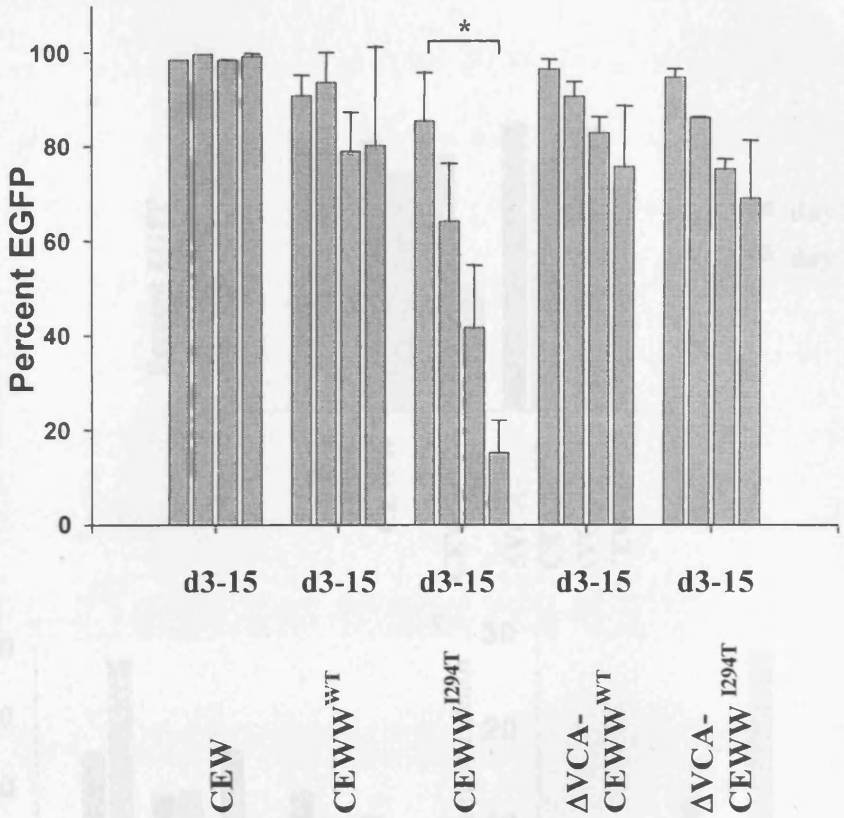


Figure 6.12. The VCA domain of mutant WASp is responsible for the toxicity seen in transduced HT1080 cells

HT1080 cells were transduced with lentiviral vectors encoding EGFP (CEW), EGFP-WASp^{WT} (CEWW) and EGFP-WASp^{I294T} (CEWW^{I294T}), both with and without the VCA domain at a multiplicity of infection of 10. Percentage of EGFP positive cells were scored at 3,6,10,15 days (represented by vertical bars) after transduction by flow cytometry, and were stable throughout the observation period for EGFP and EGFP-WASp^{WT} with no observable toxicity. In contrast, cells transduced with EGFP-WASp^{I294T} were steadily outgrown by non-transduced cells, indicating an inhibitory effect on cell growth (representative of 2-4 independent experiments). This effect is dependent on an intact VCA domain as EGFP was maintained if the VCA domain was removed. T-test *p = 0.001

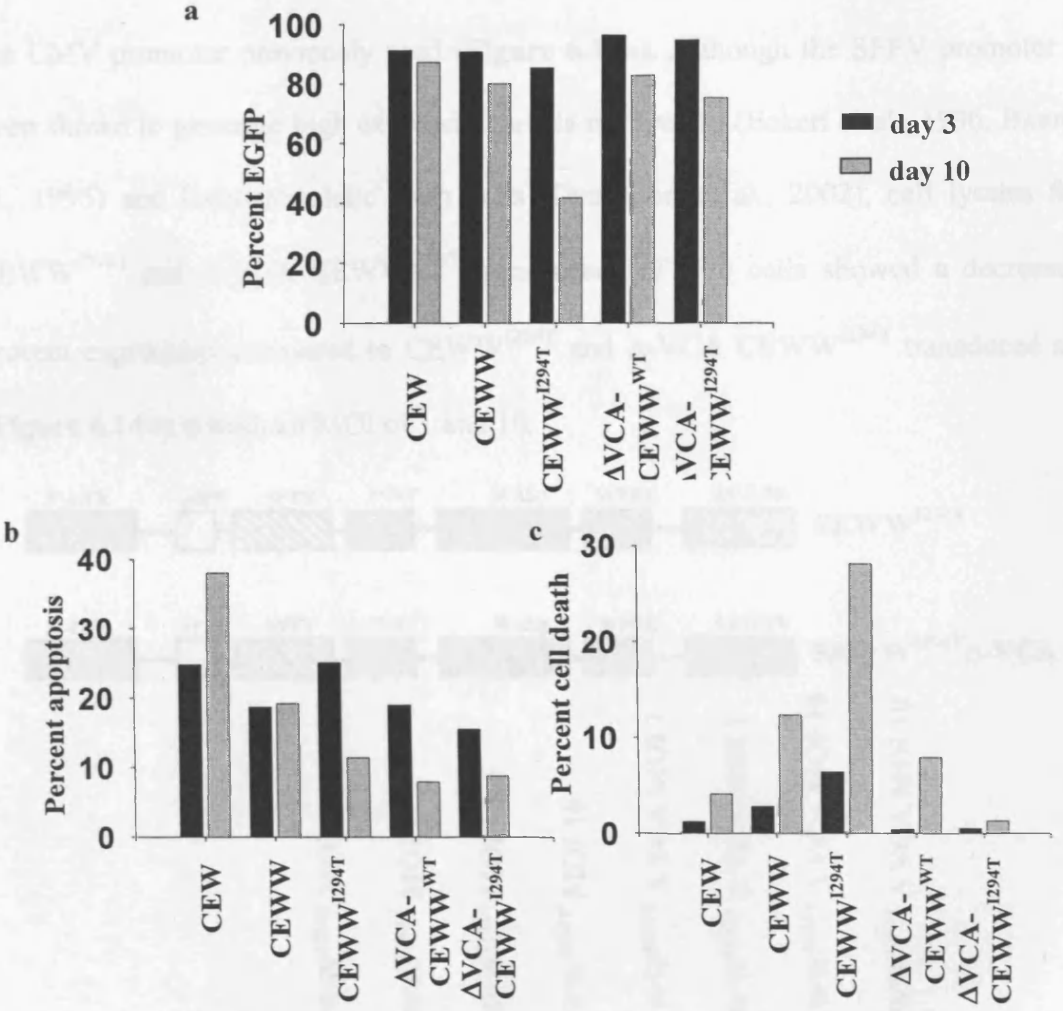


Figure 6.13 Apoptosis levels in VCA deletion mutant transduced HT1080 cells are not increased

HT1080 cells transduced with EGFP, EGFP-WASp^{WT} and EGFP-WASp^{I294T} both with and without the VCA domain were harvested from culture and counterstained with Annexin V PE and 7AAD to determine apoptosis levels. (a) EGFP levels were monitored and Annexin (b) and 7AAD (c) levels determined from the EGFP positive populations using flow cytometry. Graphs are representative of 2-4 experiments.

6.2.4 Cloning of lentiviral vectors containing an SFFV internal promoter

In order to determine cell sensitivity to the activating mutant form of WASp, lentiviral vectors were cloned that contained an SFFV 5'LTR as the internal promoter in place of the CMV promoter previously used (**Figure 6.14a**). Although the SFFV promoter has been shown to generate high expression levels in myeloid (Eckert et al., 1996; Baum et al., 1995) and haematopoietic stem cells (Demaison et al., 2002), cell lysates from SEWW^{I294T} and Δ-VCA SEWW^{I294T} transduced HT1080 cells showed a decrease in protein expression compared to CEWW^{I294T} and Δ-VCA CEWW^{I294T} transduced cells (**Figure 6.14b**) at both an MOI of 1 and 10.

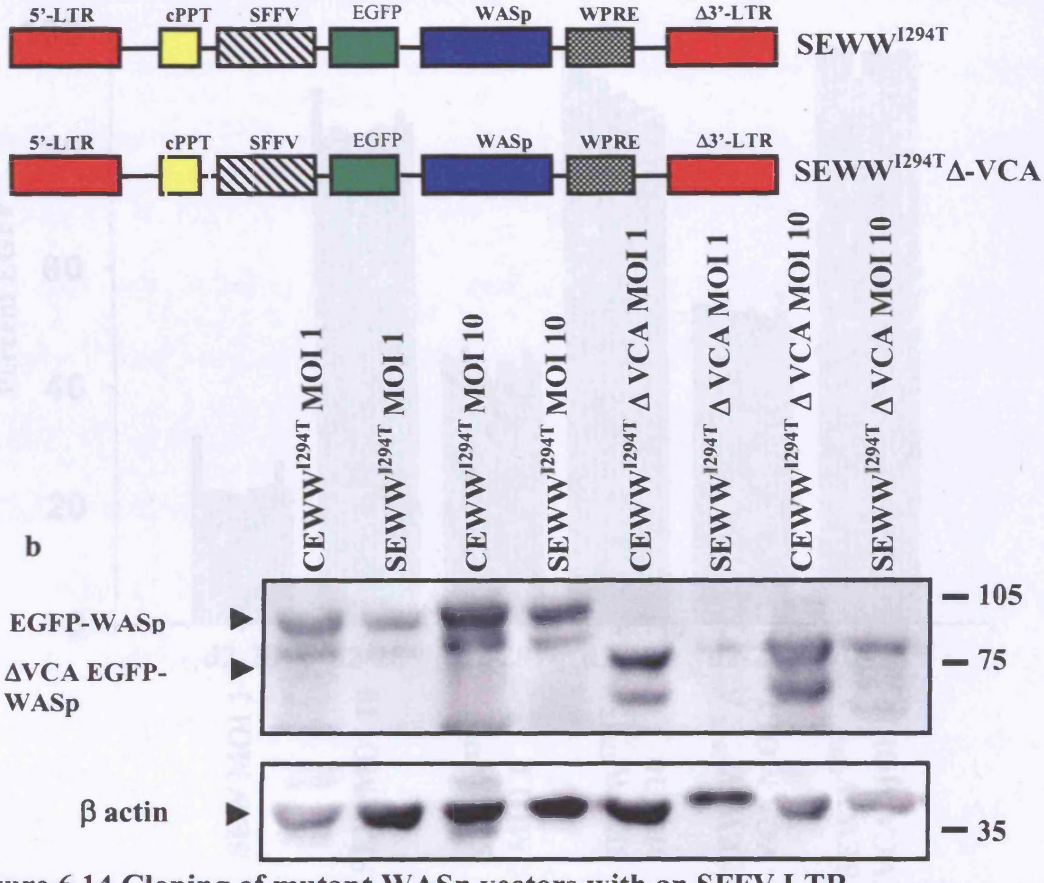


Figure 6.14 Cloning of mutant WASp vectors with an SFFV LTR
(**a**) EGFP-WASp^{I294T} and EGFP-WASP^{I294T} Δ-VCA was introduced into a HIV-1 derived self-inactivating lentiviral vector containing an SFFV 5' LTR as an internal promoter in place of the CMV previously described. (**b**) An immunoblot probing for WASp in transduced HT1080 cells revealed similar sized bands from vectors containing different promoters but reduced levels in cells transduced with SFFV containing vectors. Re-probing for β-actin was a loading control.

6.2.4.1 Viability of HT1080 cells after transduction with SFFV containing vectors.

HT1080 cells were transduced with SEW, SEWW^{I294T} and Δ-VCA SEWW^{I294T} and the viability determined by EGFP levels as described earlier. EGFP levels were maintained in SEW and Δ-VCA SEWW^{I294T} transduced cultures at both MOIs tested over a 28 day period (**Figure 6.15**). Cells transduced with SEWW^{I294T} at a low MOI also maintained their EGFP levels. Upon transduction with a higher MOI there was death of transduced cells with 10% of transduced cells dying (**Figure 6.15**). This cell death was at a greatly reduced rate and level to that observed previously with vectors containing a CMV promoter.

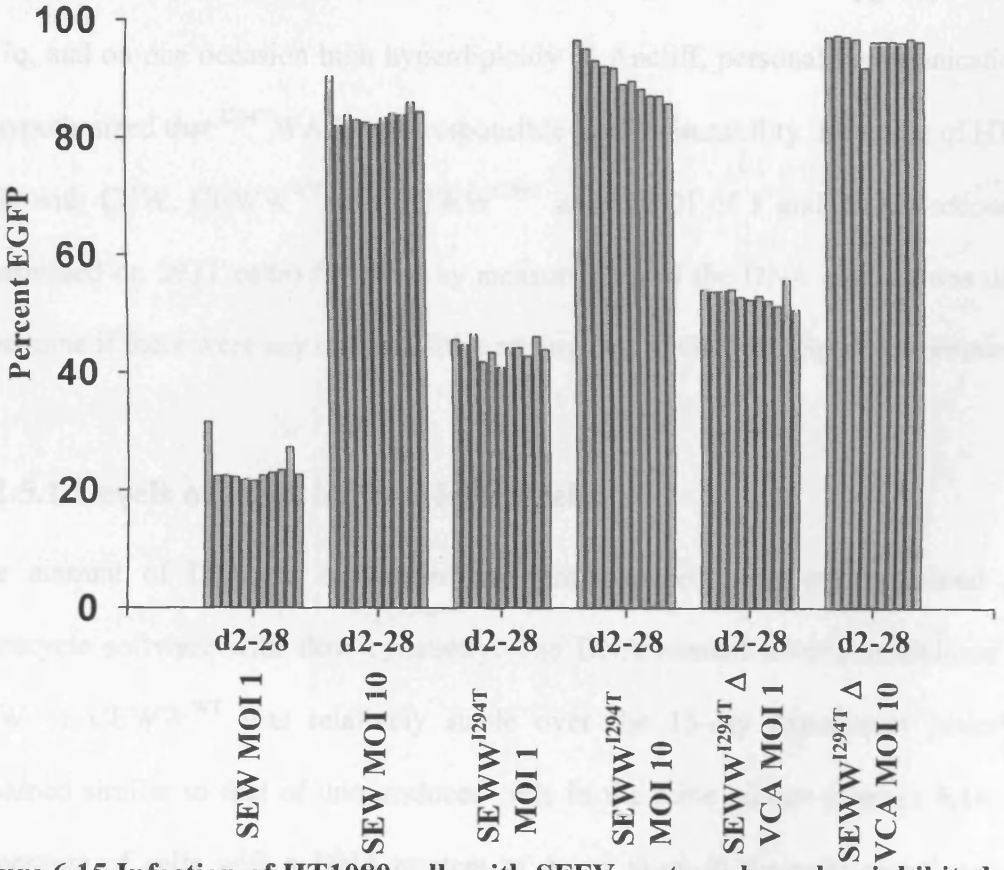


Figure 6.15 Infection of HT1080 cells with SFFV vectors shows less inhibited growth of EGFP-WASp^{I294T} expressing cells

HT1080 cells were transduced with lentiviral vectors encoding EGFP (SEW) or EGFPWASp^{I294T} (SEWW^{I294T}) with and without the VCA domain at a multiplicity of infection of 1 and 10. Percentage of EGFP positive cells were scored at 2-28 days (represented by vertical bars) after transduction by flow cytometry, and were stable throughout the observation period for EGFP with no observable toxicity. At low doses there was no toxicity seen with EGFP-WASp^{I294T} but was seen at high doses although at a much reduced level and rate than previously seen with CMV containing vectors. This indicates the inhibitory effect of I294T is dose dependant.

The viability of transduced cells was again measured by annexin and PI staining to determine the levels of apoptosis and cell death. There was little detectable difference in Annexin or PI levels between samples at both MOIs tested (data not shown). Thus the cell death observed in SEWW^{I294T} transduced cells by loss of EGFP was small and undetectable compared to SEW and Δ-VCA SEWW^{I294T} transduced cells, again different to that previously observed in CEWW^{I294T} cells.

6.2.5 WASp^{I294T} induces genomic instability and polyploidy

Cytogenetic analysis of bone marrow samples on three separate occasions from the index patient revealed variable abnormalities including loss of heterozygosity, inversion of 7q, and on one occasion high hyperdiploidy (P Ancliff, personal communication). It is hypothesized that ^{I294T}WASp was responsible for this instability. Infection of HT1080 cells with CEW, CEWW^{WT} and CEWW^{I294T} at an MOI of 1 and 10 (infectious titer determined on 293T cells) followed by measurement of the DNA content was used to determine if there were any abnormalities arising from EGFP-WASp^{I294T} expression.

6.2.5.1 Levels of DNA in transfected cells

The amount of DNA in transduced and untransduced cells was measured using multicycle software with flow cytometry. The DNA content of cells transduced with CEW or CEWW^{WT} was relatively stable over the 15-day experiment period and remained similar to that of untransduced cells in the same culture (**Figure 6.16**). The percentage of cells with a DNA content of 4n or more in the cells transduced with CEWW^{I294T} increased for the duration of the experiment (**see Figure 6.16 inset for 4n+ determination**) at an MOI of both 1 and 10 relative to untransduced cells in the same culture (**Figure 6.16**). In fact there was a 6-fold increase in the percent of cells with 4n

or more DNA levels in the cells transfected with EGFP-WASp^{I29T} compared to untransfected cells. This effect was more pronounced in the cells infected at an MOI of 10 (**Figure 6.16**). Using linear gates to quantify the percent of cells at each stage in mitosis the percent of cells with 4n as well as an increase in 4n can be seen (**Figure 6.17a**).

Samples from the cultures were obtained and cytospun onto slides. These were stained histochemically and visualised by light microscopy. Cultures transduced with CEW or CEWW^{WT} showed predominantly cells with single nuclei. In contrast cells transduced with CEWW^{I294T} showed a high number of cells with multiple nuclei (**Figure 6.17b**) confirming the results obtained by flow cytometry.

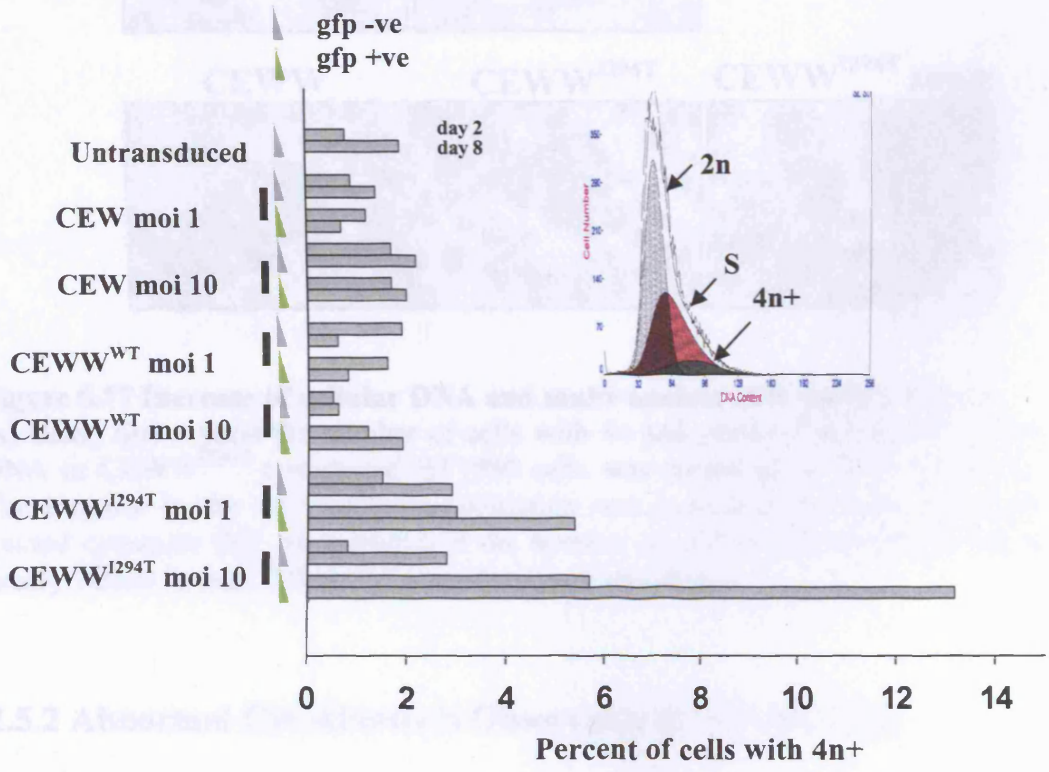


Figure 6.16 Analysis of DNA content in EGFP+ and EGFP- cell populations
HT1080 cells were transduced with lentiviral vectors encoding EGFP, EGFP-WASp^{WT} and EGFP-WASp^{I294T} at an MOI of 1 and 10. The cells were ethanol fixed before staining with PI and the cellular DNA content of EGFP+ and EGFP- cells measured by flow cytometry. Multicycle software (see inset) was used to determine the relative DNA content at day 2 and day 8. Cells transduced with EGFP-WASp^{I294T} showed an increase in their DNA content compared to EGFP or EGFP-WASp^{WT}.

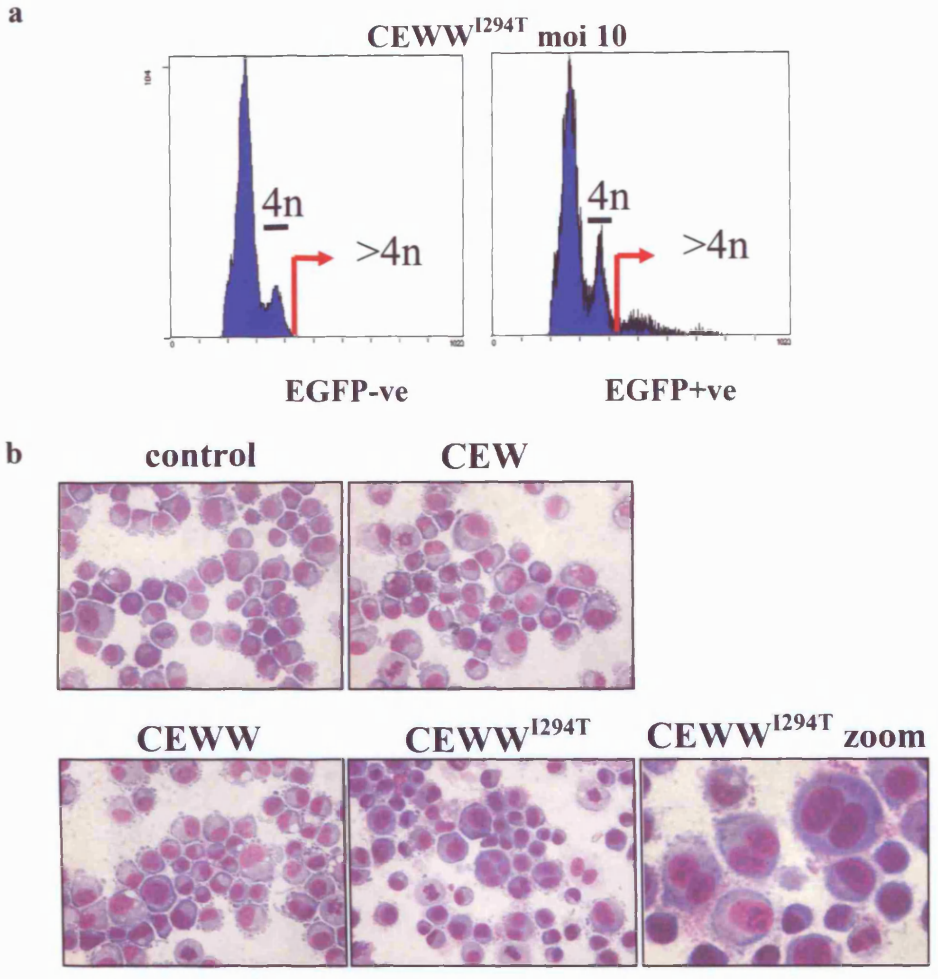


Figure 6.17 Increase of cellular DNA and multi-nuclear cells during mitosis
(**a**) Using linear gates the number of cells with 4n and greater than 4n (red arrow) DNA in CEWW^{I294T} transduced HT1080 cells was measured by flow cytometry. The increase in the 4n EGFP+ve population was confirmed by histochemically stained cytospins (**b**). An increase in the number of cells with multiple nuclei is clearly visible in the CEWW^{I294T} transduced cell population

6.2.5.2 Abnormal Cytokinesis is Observable in HT1080 cells

To assess why cells were appearing with multiple nuclei after a short period of culture HT1080 cells were transfected at an MOI of 10 with CEW, CEWW^{WT} and CEWW^{I294T} and after 24 hours, transferred to coverslips at a low density of 10^3 per coverslip. They were then allowed to proliferate for a further 24 hours so cytokinesis could occur,

before being fixed and stained for actin and microtubules. Cells infected with CEW grew normally and cells undergoing cytokinesis could be spotted due to the presence of a contractile actomyosin ring where the daughter cell is being cleaved off (**Figure 6.18a**). This ring was seen at a frequency of 12% (**Figure 6.18b**) for CEW transduced cells. However this was impaired in cells transduced with CEWW^{WT} (6%) and virtually absent in cells transduced with CEWW^{I294T} (0.6%) (**Figure 6.18b**). With this lack of actomyosin ring a corresponding disrupted pattern of actin staining compared to CEW transduced cells was seen (**Figure 6.18a**) showing the effect EGFP-WASp^{I294T} was having upon the cells.

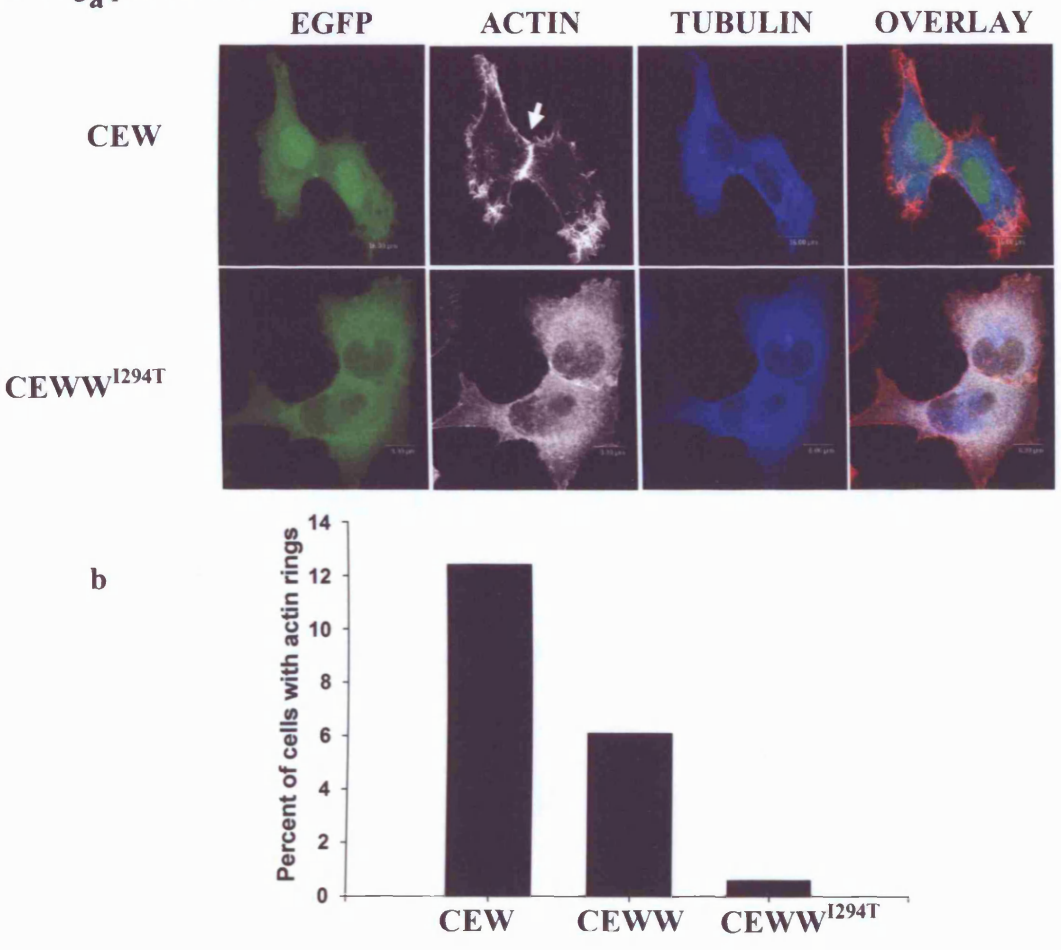


Figure 6.18 Loss of actin contractile rings in dividing HT1080 fibroblasts
HT1080 cells were transduced with EGFP, EGFP-WASp^{WT} or EGFP-WASp^{I294T}, plated at low density onto glass coverslips and allowed to grow overnight. The transduced cells (green) were stained for F-actin (white/red) and tubulin (blue) and images captured by confocal microscopy. (**a**) Contractile rings in dividing cells can be seen in EGFP transduced cells (arrow) but are lost in EGFP-WASp^{I294T} transduced cells with a corresponding increase in cortical F-actin.(**b**) The total percent of cells with contractile rings is reduced for both EGFP-WASp^{WT} and EGFP-WASp^{I294T} compared to EGFP alone. Total cells counted was 150-200

6.3 Discussion

In this chapter a novel mutation of WASp has been investigated that does not lead to a classical WAS phenotype. In fact this mutation led to disruption of the auto inhibited confirmation and consequently to dysregulation of actin polymerisation. In patient primary macrophages this manifests itself as increased clusters and abnormal distribution of podosomes that have increased levels of F-actin judged by fluorescence intensity of confocal images. This dysregulation of actin is further shown by the lack of phagocytosis and oxidative burst in granulocytes upon physiological stimulation. The loss of the protein tertiary structure can be attributed to the introduction of a polar side chain into the alpha helix of the C-terminus that would normally fit into the hydrophobic core of the GBD of WASp. A phosphomimicking mutant ^{Y291E}WASp (Cory et al., 2002) and the recently discovered ^{L270P}WASp (Devriendt et al., 2001) are adjacent and opposite respectively to ^{I294T}WASp and have similar properties of instigating actin dysregulation. This suggests the disruption of the auto inhibited confirmation may be similar to Y291 phosphorylation which has been shown to be important in the direct regulatory function of WASp (Cory et al., 2002).

A lentivirus containing wild-type EGFP-WASp was mutated to contain EGFP-WASp^{I294T} and a pattern of expression similar to wild type was seen in non-haematopoietic cell lines. A western blot of transfected cell lysate revealed a mobility shift on SDS-PAGE with ^{I294T}WASp, which has also been seen in patient cells. It is unclear as to why this shift occurs but it may be due to post-translational modifications at sites previously unavailable in an autoinhibited WASp or directly related to the presence of the threonine, which can be glycosylated.

The dysregulation of actin is shown *in vivo* through the formation of podosomes in EGFP-WASp^{I294T} transduced Bac-1 macrophages, which do not normally make podosomes. Furthermore they are formed within the cells in a manner similar to that presented in primary macrophages, with multiple clusters and ring like structures. This could be occurring because the active confirmation favoured by ^{I294T}WASp is making Y291 more accessible to kinases and phosphatases, thus enhancing the effect of WASp through Cdc42, which has been shown to be important in podosome assembly and disassembly (Burns et al., 2001b; Linder et al., 1999).

^{I294T}WASp results in monocytopaenia and neutropaenia and bone marrow cells fail to proliferate *in vitro* (P.Ancliff, in press). In addition we have seen an increase in spontaneous apoptosis in bone marrow, in both mature and progenitor populations and in inducible apoptosis in peripheral blood lymphocytes compared to normal. This shows that dysregulation of the actin cytoskeleton has an adverse effect on cell survival.
The adverse effect of ^{I294T}WASp on cell survival is further supported by the cell death seen in HT1080 cells at high and low MOI's when a strong promoter is used. Over a 14-day period untransduced cells outgrew the transduced cells and when apoptosis measured in the EGFP positive population, it was increased.

To confirm the cell death seen was due to actin dysregulation a further modification was made to remove the VCA region of WASp. When expressed in cells the mobility shift on SDS-PAGE was still seen showing any modifications that result in the shift are not in the VCA domain. The EGFP expression in Δ-VCA CEWW^{I294T} transduced cells was maintained at a similar level to that seen with CEWW^{WT} transduced cells throughout the 15 day experiment and there was no corresponding increase in apoptosis or cell death

showing the actin dysregulation is responsible for the apoptosis. Transduction with vectors utilising an SFFV promoter, which was shown by immunoblot to give reduced expression in HT1080 cells also lead to cell death but at a much reduced level and rate. This suggests that the level of expression of EGFP-WASP^{I294T} within the cell is critical to induce the deleterious effect.

A low level of toxicity was seen in HT1080 cells transduced with EGFP-WASp^{WT} but was not reproducible. We have previously shown overexpression of EGFP-WASp^{WT} has been associated with toxicity in transduced human dendritic cells and macrophages where the actin cytoskeleton has been severely disrupted (Chapter 3 and 4). This was not reduced upon removal of the VCA domain, therefore there may be other physiological activators and adaptors e.g. PIP_2, Nck, Hck, Cdc42, WIP, important in the regulation of WASp, which are contributing to the adverse effect of WASp over expression. Again this seems to be expression related as the lower levels seen when an SFFV promoter was used did not have the same effect.

To try and determine the underlying reason for the deleterious effect of WASp^{I294T} in patient primary cells and overexpression of EGFP-WASp^{I294T} in transduced HT1080 cells analysis of the DNA content of the cells was performed. Cytogenetic abnormalities detected in bone marrow samples from the patient are indicative of genomic instability. This effect is replicated by the emergence of polyploidy, loss of heterozygosity and amplification of *Rb* in mutant lymphoblastoid cell lines (P. Ancliff, personal communication). In addition the polyploidy observed in HT1080 cells expressing EGFP-WASp^{I294T} also suggests genetic instability. This may be a reason why there is neutropaenia and monocytopaenia in the patient.

Cytokinesis requires the regulated assembly of a contractile ring of actin and myosin that cleaves the cell in two (Glotzer, 2001). In fission yeast *Schizosaccharomyces pombe*, this forms in the middle of the cell early in mitosis and contracts after anaphase. The equatorial location of the actomyosin ring is maintained by late-mitotic microtubular structures. If these are disrupted, the position of the ring drifts resulting in asymmetric cell division and genomic instability (Pardo and Nurse, 2003). Also in yeast, it is believed that the cortical actomyosin ring plays an important role in the establishment of spindle orientation through interaction with astral microtubules that form on the spindle pole bodies before ring formation (Gachet et al., 2001). Pharmacological inhibition of actin polymerisation, results in activation of a mitotic checkpoint that ensures that mitotic spindles are correctly orientated before anaphase is allowed to take place (Gachet et al., 2001). These findings in yeast therefore point to a mechanism for growth arrest and genomic instability in cells that have dysregulated actin dynamics through expression of $WASp^{I294T}$ in primary cells and EGFP-$WASp^{I294T}$ in culture.

The actin polymerising activity in the actomyosin ring is highly dynamic, resulting in continuous assembly and disassembly, and in fission yeast depends on the Arp2/3 complex forming, Cdc12, profilin and WASp (Pelham and Chang, 2002). In WASp mutant yeast cells (*wsp1*Δ), the closure of the actin ring is significantly reduced. In EGFP-$WASp^{I294T}$ transduced HT1080 cells undergoing cell division there is a loss of the actomyosin ring corresponding with an increase in the cell ploidy. It is therefore likely that dysregulated WASp activity could compromise cytokinesis either directly by disturbing normal ring formation, or indirectly by depletion or sequestration of critical substrates for actin polymerisation in cytoskeletal structures. One of these substrates

could be Cdc42. WASp and Cdc42 interact in the activation of WASp (Aspenstrom et al., 1996; Kolluri et al., 1996; Symons et al., 1996) and the transduction of signals from the cell surface. Recently it has been shown that over expression of Cdc42 in cells in culture leads to polyploidy and growth arrest (Muris et al., 2002) mimicking what has been observed here.

Insights into problems associated with overexpression of mutant WASp and to some extent wild-type WASp are useful for elucidating viable gene therapy protocols. When a protein with the complexity of WASp is restored into the haematopoietic compartment, there may be a higher degree of regulation needed than for other primary immunodeficiencies such as X/SCID where expression from a viral promoter does not seem to have any deleterious effects (Cavazzana-Calvo et al., 2000a; Gaspar et al., 2004). The complexity of the effects WASp can exert can be seen in HT1080 cells. Expression from a CMV promoter can lead to cell death, but this is not seen when an SFFV promoter is used.

CHAPTER 7

DISCUSSION

Discussion

Gene therapy for primary immundeficiencies using retroviral vectors has moved from the laboratory to the clinic in the past few years with great success for SCID-X1 (Cavazzana-Calvo et al., 2000a; Hacein-Bey-Abina et al., 2002; Gaspar et al., 2004) and ADA-SCID (Aiuti et al., 2002)(Gaspar *et al* ASGT 2005). The success of SCID-X1 is thought in part to be attributable to the massive selective advantage for cells containing a functional common γ chain (Stephan et al., 1996), in addition to advances in vector design and transduction protocols.

Wiskott-Aldrich syndrome is thought to be a good candidate for gene therapy due to the poor prognosis with a mismatched bone marrow transplant (reviewed in (Thrasher and Kinnon, 2000b)) and the reported selective advantage seen when somatic mosaicism occurs in patients (Wada et al., 2001; Wada et al., 2003; Wada et al., 2004) and in competition assays in a murine model (Strom et al., 2002). In fact using retroviral vectors we (Chapter3) and others (Klein et al., 2003; Strom et al., 2003b) have shown successful correction of a murine model with WASp expression.

Clinical gene therapy trials have not been without problems. Insertional mutagenesis, a predicted but thought to be rare possibility, has occurred leading to complications in 3 patients treated for SCID-X1 (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). In fact it has now been established that retroviruses prefer to integrate close to a gene transcriptional start site (Wu et al., 2003) and the number of insertional events have been linked to the frequency of mutagenic events in murine models (Modlich et al., 2005). In addition it has failed where older patients have been treated (Thrasher et

al., 2005) which has been postulated to be linked to patient age and an effective thymic microenvironment.

Problems with oncoretroviral gene therapy also include the well documented need for mitosis before any insertions can occur in the genome (Miller et al., 1990b). This was the rationale behind the development of lentiviral vectors, mainly based upon HIV-1. Lentiviral based vectors have been modified to be self-inactivating, thus removing enhancer regions thought to be important in insertional mutagenesis (Zufferey et al., 1998), and reduces gene silencing (reviewed in (Ellis, 2005), although recently full length transcripts were detectable by PCR (Logan et al., 2004b). Elements have been included in the vectors to increase expression and insertion into non-dividing cells and have internal promoters to enhance gene expression. Unfortunately they have a propensity to integrate into active genes (Schroder et al., 2002; Mitchell et al., 2004) compared to transcription start sites for MLV based retroviruses. This may cause problems similar to that seen in the Paris clinical trial (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b) Interestingly avian sarcoma viral vectors do not show any propensity to insert either in active genes or at transcription start sites (Mitchell et al., 2004), possibly increasing the safety of such vectors. Other viral vectors able to integrate into non-dividing cells are proving to be efficient delivery vectors. These include EIAV (O'Rourke et al., 2005; Siapati et al., 2005) and foamy virus (Hirata et al., 1996; Josephson et al., 2002; Leurs et al., 2003). The insertion patterns of these vectors need to be investigated to determine if active genes or transcriptional start sites are also preferred. It may be decided that site directed or site specific integration into a piece of redundant DNA is required. This has been demonstrated using bacteriophage recombinases (Groth et al., 2000) and work is on-going using site specific

endonucleases, homologous recombination and AAV and is reviewed in (Glover et al., 2005).

We have shown successful transduction and correction of WAS knockout dendritic cells, macrophages and murine stem cells, capable of reconstituting a murine knock out model, with lentiviral vectors (Chapters 4+5), to levels similar to that observed in other groups (Charrier et al., 2005; Dupre et al., 2004), lending much promise to the development of a clinical trial for WAS utilising lentiviral vectors. The use of endogenous WAS promoter sequences to express WASp in a lentiviral gene therapy model has been shown (Chapters 4 + 5), and compares favourably with reconstitution in T cells shown with the 1.6Kb promoter (Dupre et al., 2004) and the 500bp promoter (Martin et al., 2005) and with expression derived from a viral LTR in this study. In this study the protein expression levels, determined by EGFP, was always lower than that seen for EGFP alone. This could be due to high level expressing cells dying, leaving low level expressers as viable. In fact some toxicity was seen when normal cells containing WASp had EGFP-WASp introduced (Chapter 3), and toxicity from the I294T mutant WASp was increased with expression levels (Chapter 6). The presence of EGFP tagged onto WASp cannot be excluded from being the cause of problems associated with high level expression, therefore use of an IRES sequence to separate expression of WASp and a marker gene may be useful to exclude this possibility. More normally regulated WASp expression levels may be attained by these endogenous sequences reducing toxicity associated with high level expression. WASp expression is restricted to the haematopoietic lineage (Derry et al., 1994; Stewart et al., 1996), which is thought to be dictated by regulatory elements found upstream of *WAS* and within the sequences used as promoters in this study (Hagemann and Kwan, 1999; Petrella et al.,

1998). Haematopoietic restricted expression is an attractive proposition for immunodeficiencies as expression of haematopoietic–specific genes in non-haematopoeitic tissue may result in undesired side effects leading to insertional mutagenesis (reviewed in (Baum and von Kalle, 2003). Although expression can be attained from both the 500bp and 1.6 Kb WASp promoters, further investigation into the active elements within the 1.6Kb sequence and transcription factors responsible for the haematopoietic restricted expression is required. A transgenic mouse model where EGFP is expressed under the control of the 500bp or 1.6Kb promoter sequence would reveal expression temporally and spatially, thus helping with the understanding of expression patterns and whether or not the promoters are actually haematopoietic specific.

In this study, recovery of podosomes was used as a measurement for efficiency and efficacy of WASp reconstitution based upon podosome recovery when *WAS* is microinjected into WASp null dendritic cells (Burns et al., 2001b) and the findings by Linder *et al* that XLT patients with a lower level of WASp have podosomes present but in reduced numbers (Linder et al., 2003). Other groups have also used podosome recovery (Charrier et al., 2005) but many have focused upon recovery of T cell function using recovery of T cell proliferative responses and CD3 capping (Wada et al., 2002; Klein et al., 2003), IL-2 secretion (Strom et al., 2003a) or immunity to viral infections (Strom et al., 2003b). Although some T cell proliferative recovery was observed in this study further study of the immune system recovery is needed to determine if gene therapy for *WAS* is a future clinical treatment.

WASp is linked to actin dynamics, therefore podosome turnover in reconstituted cells may reveal whether actin dynamics are normalised. Chemotaxis is defective in WAS null cells *in vitro* (Badolato et al., 1998; Zicha et al., 1998) and *in vivo* (de Noronha et al., 2005; Snapper et al., 2005), therefore recovery of chemotaxis when WASp is introduced would show effectiveness of gene transfer. In addition the recovery *in vivo* of splenic and lymph node architecture, shown to be defective in WASp mice (de Noronha et al., 2005), would be indicative of normal migration, immune fuction and ability to respond normally to antigen. The inability to mount a normal inflammatory response in WASp KO mice could be exploited using contact sensitivity to DNFB (Del Prete et al., 2004) or inability to class switch in response to low concentrations of dextran (Westerberg et al., 2005). Another defect, phagocytosis, could be measured *in vitro* using immunoglobulin coated beads (Lorenzi et al., 2000a) or *in vivo* using apoptotic cells (Leverrier et al., 2001b). An increase in inflammation or phagocytosis in reconstituted mice would suggest successful recovery of phenotype.

Due to the complex interactions of WASp within cells, regulation of expression is important. Insights into the effects of WASp over expression have been seen in our studies on a novel I294T mutant of WASp, utilising the lentiviral gene delivery system. Dysregulation of the actin cytoskeleton in patient cells, caused by the mutant WASp expression, lead to severe neutropaenia and monocytopaenia, a lack of immune cell function, increased apoptosis and genomic instabilities including polyploidy. In HT1080 cells expression of mutant WASp lead to death of transduced cells, actin dysregulation, increased apoptosis and defects in cytokinesis, leading to polyploidy, mimicking the patient symptoms. Interestingly there was some death associated with high level expression of wild-type WASp in HT1080 fibroblasts (Chapter 6) but was not

reproducible. Furthermore it was not seen when a SFFV promoter was used. This is thought to be due to lower levels of protein expression. Further work is needed to fully elucidate the mechanism through which the mutant WASp exerts its cellular effects and the effects of protein concentration. Removal of the VCA domain and with it the actin polymerisation domain abrogated the toxicity of the I294T mutant WASp. This may not be the best control however as removal of the entire domain also would affect the folding of the protein and therefore the auto inhibition. An improved method for removal of the actin polymerization ability could be through the mutation of amino acid 470 in WASp. It has been shown that the point mutation of L470A abrogates actin polymerisation ability by not allowing the ARP2/3 complex to undergo a conformational change upon binding to WASp (Goley et al., 2004; Rodal et al., 2005). WASp folding should still be normal with this mutation therefore showing if the I294T mutant exerts all its effects through excess actin polymerisation, in addition to determining whether possible toxicity due to overexpression of wild-type WASp is also due to excess actin polymerisation.

As WASp is haematopoietic restricted, repetition of the I294T WASp experiments on a haematopoietic cell line e.g. U937 or Jurkats, would be useful to see if the same effects would be observed. In addition primary cells such as CD34 stem cells could be transduced and the effects on cell division, apoptosis and genomic stability measured. It would be predicted to exert the same cytokinesis defects in haematopoietic cells. It has been shown WIP is tightly involved in the regulation of WASp expression (Ramesh et al., 1997; Stewart et al., 1999; Luthi et al., 2003), preventing degradation of WASp by calpain (Shcherbina et al., 2001) thereby controlling the cellular level of WASp. This may prevent over expression in haematopoietic cells negating the effect of the auto-

active WASp. The interaction of the mutant WASp with cellular binding partners also needs to be investigated, especially Cdc42 as there has been some recent data on levels of Cdc42 during cytokinesis and the effects of increasing or reducing the levels during cytokinesis (Oceguera-Yanez et al., 2005). The sensitivity of cells to WASp expression levels is interesting as it may be pertinent to gene therapy protocols. High level expression may not be the most efficacious in the long term, further increasing the potential of endogenous promoter sequences which may give a more normal protein expression level.

Further investigation into mutant WASp could be performed *in vivo*. This includes transducing lineage negative stem cells and infusing into lethally irradiated recipients in a manner similar to the gene therapy protocol described in Chapter 6. Pressure upon transduced cells to divide to repopulate the murine haematopoietic compartment may lead to genomic instabilities, seen in the patient. Alternatively the transduced cells may disappear altogether due to toxicity, as seen in the HT1080 fibroblasts. Generation of a knock in mutant model where I294T WASp is expressed in the place of wild-type may also further elucidate the effects of the mutant upon cell division and its subsequent immunological phenotype.

The future prospects for retroviral gene therapy look bright. However there are some areas where improvements need to be made and are currently being investigated.

Improved vector design is one such area. SIN vectors for retroviral vectors are most likely to be developed for clinical use before the use of lentiviral vectors. They are thought to be safer due to the loss of enhancer activity, although full length genomic

transcipts are detectable (Logan et al., 2004a), and may also reduce silencing (reviewed in (Ellis, 2005). Silencing of integrated virions occurs in both retrovirally and lentivirally transduced cells leading to increased copy number to gain sufficient cellular expression levels and with it an increased risk of insertional mutagenesis based upon a multiple hit theory. Use of *cis*-elements such as locus control regions (LCRs), chromatin insulators and scaffold/matrix attachment regions (SMARs) are being investigated for their ability to reduce silencing. LCRs upstream of the B-globin cluster have been used *in vitro* (Lotti et al., 2002) and *in vivo* (May et al., 2002), in lentiviral vectors, to show antisilencing effects. Insulators protect gene expression from neighbouring enhancers or silencers (reviewed in (Burgess-Beusse et al., 2002) thus preventing positional effects of the integrated virion. S/MARs are mammalian origins of replication that also have some insulating activity (reviewed in (Conese et al., 2004). They have been used successfully, in a nonviral setting, to enhance persistence of gene expression over long periods of time (Ehrhardt et al., 2003) by replicating when there is cell division. Incorporation of these elements into lentiviral vectors, including non-integrating lentiviral vectors, already shown to allow long-term expression in non-dividing tissues (Yanez-Munoz et al., 2006) may improve expression from low integrated copies of virus and non-integrated virus thus reducing the chance of insertional mutagenesis.

Improvements in the purification and time to transduce stem cells, and the mechanisms behind successful bone marrow repopulation may also improve and increase the safety of gene therapy protocols as fewer cells may be needed to be transduced and infused into the recipients.

In conclusion, Wiskott-Aldrich syndrome is a good candidate for correction by gene therapy, but further investigation into the possible detrimental effects of high level expression is needed. In addition lentiviral vectors have been shown to be excellent gene delivery vehicles and should be considered for future clinical trials in place of oncoretroviral vectors. Their capacity to include complex regulatory elements and internal promoters, possibly endogenous, should allow tighter regulation of expression thus improving the safety concerns following mutagenic events with existing oncoretroviral trials.

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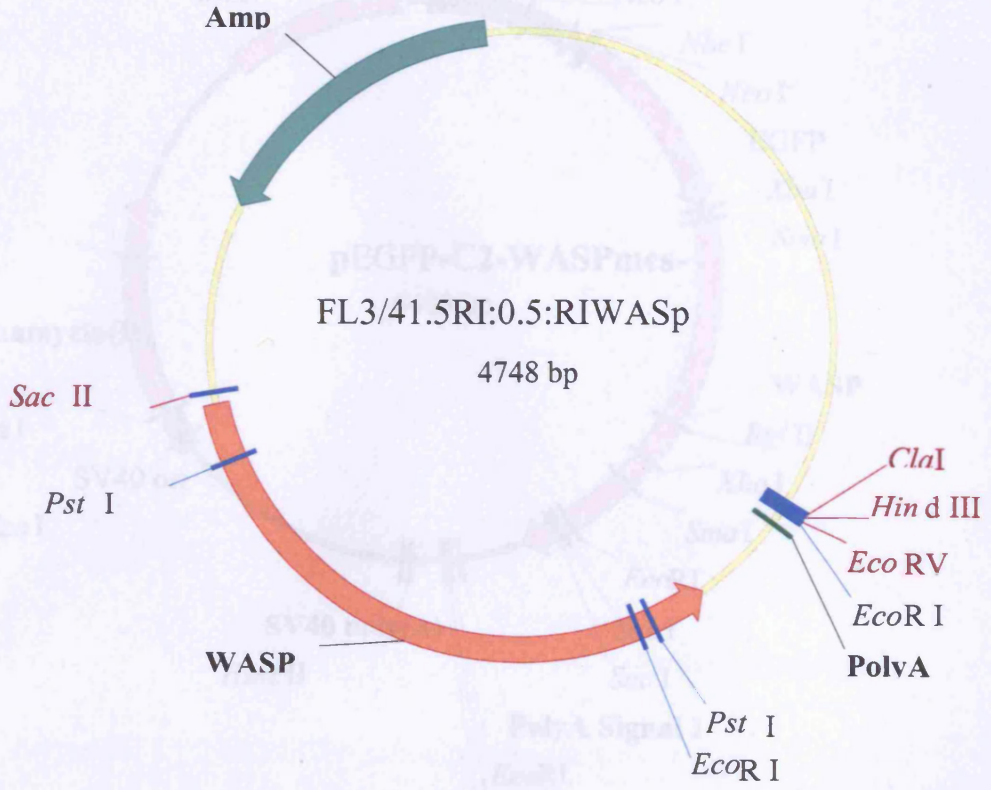
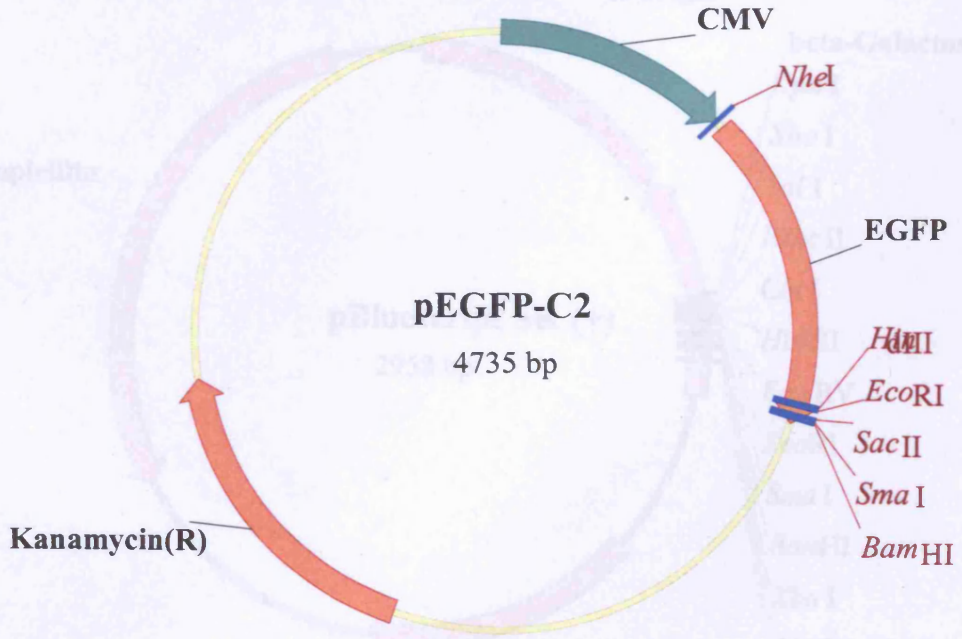
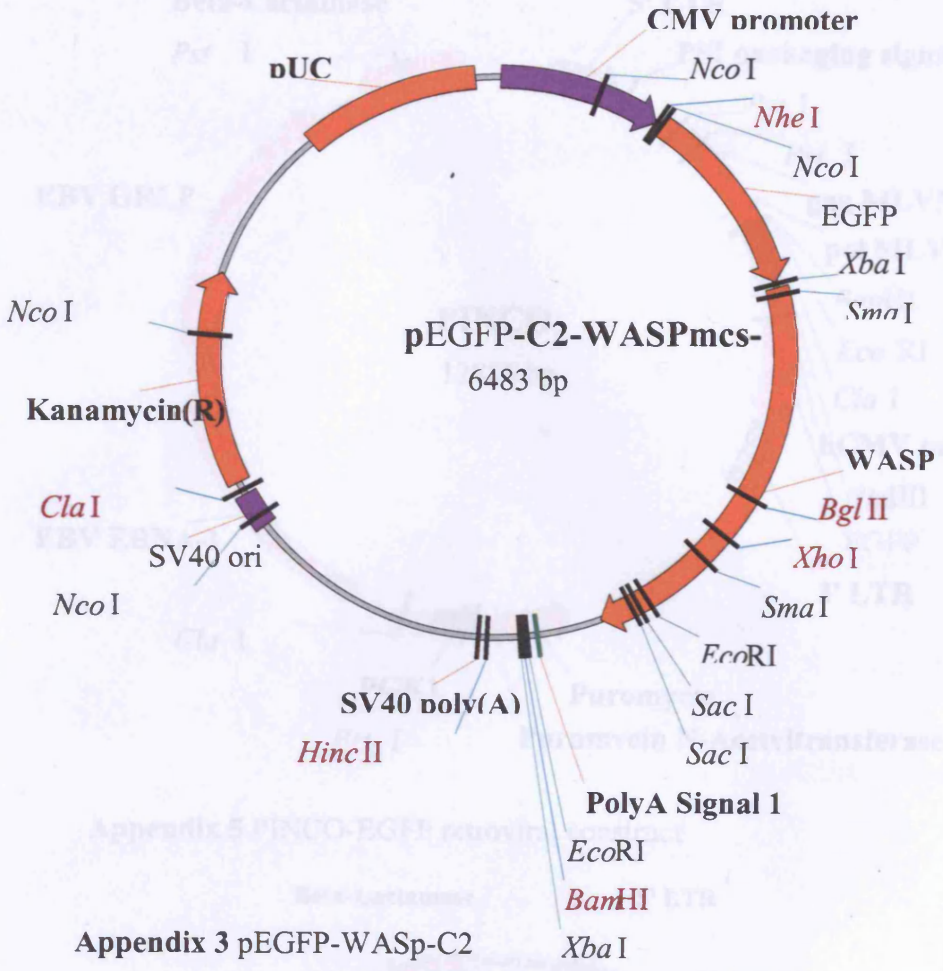
Appendix



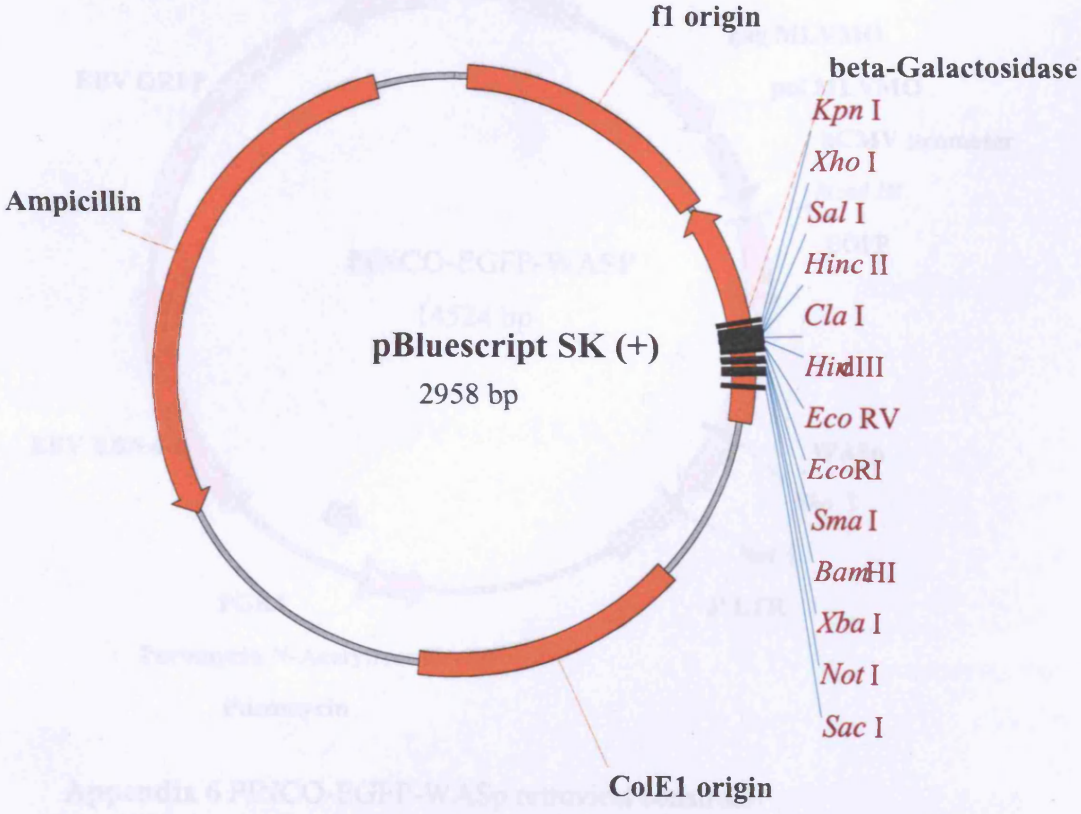
Appendix 1 FL3/41.5RI:0.5RI. Plasmid containing WASp cDNA. (gift from J.Derry)



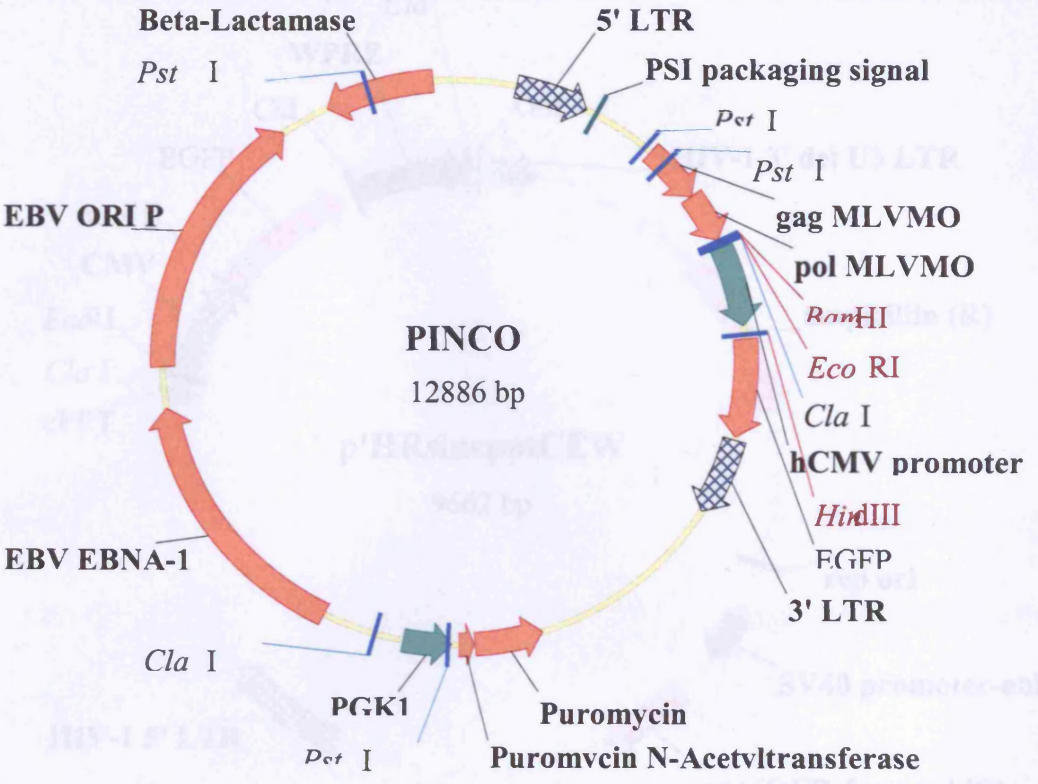
Appendix 2 pEGFP-C2 (Clontech)



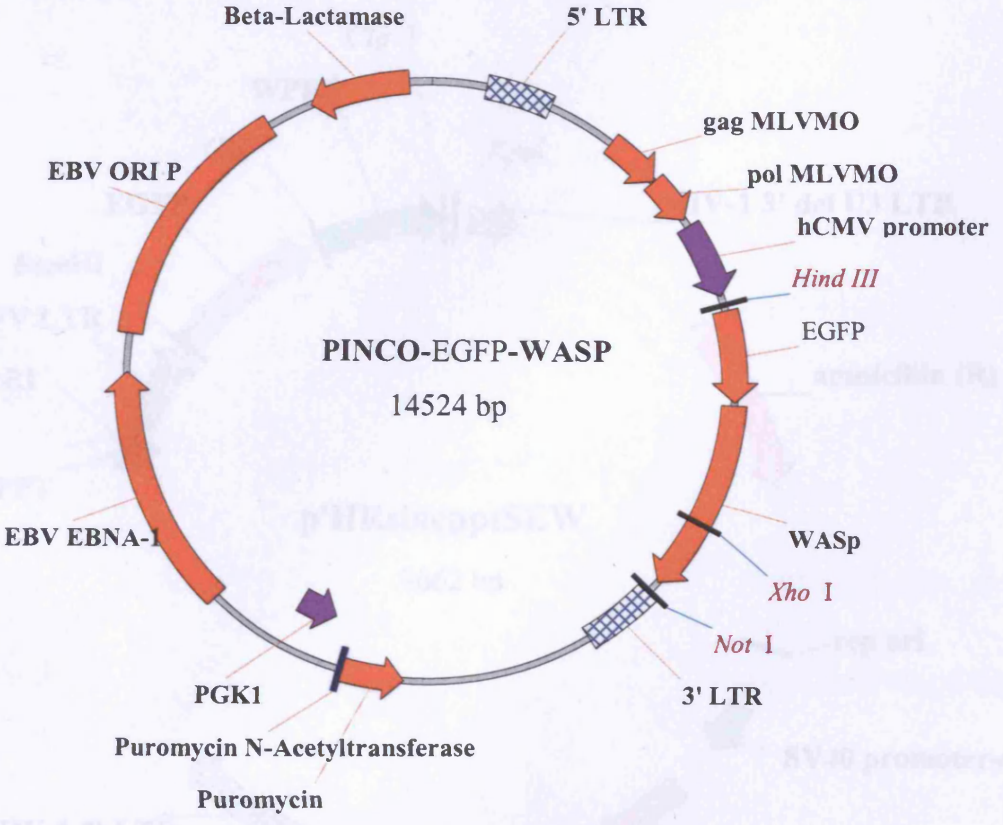
Appendix 3 pEGFP-WASp-C2



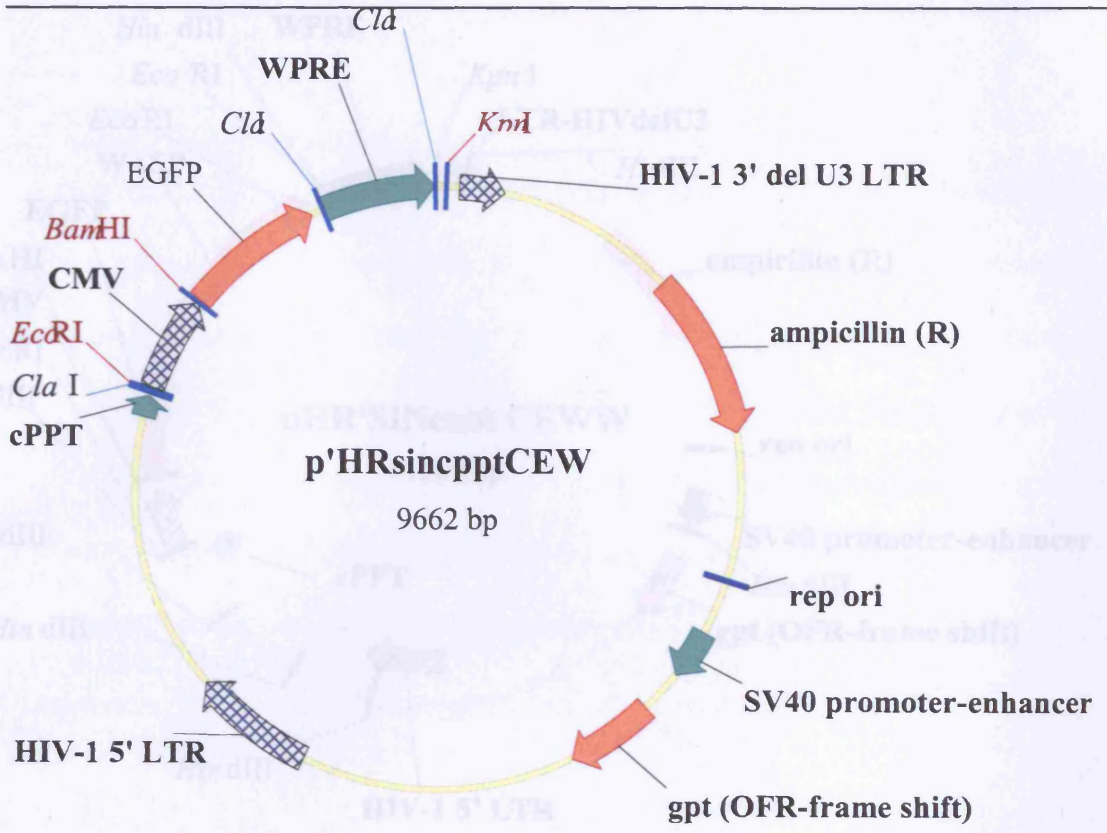
Appendix 4 pBluescript



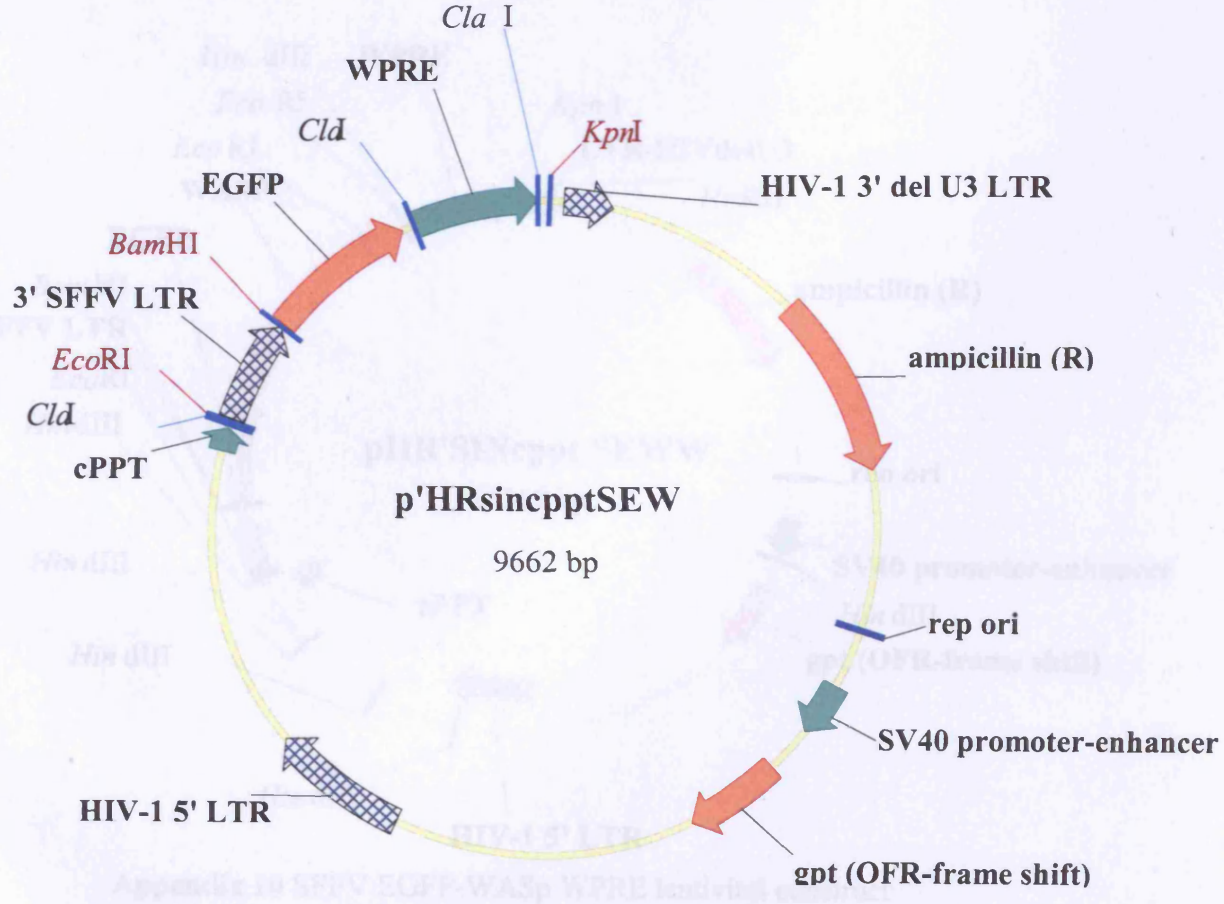
Appendix 5 PINCO-EGFP retroviral construct



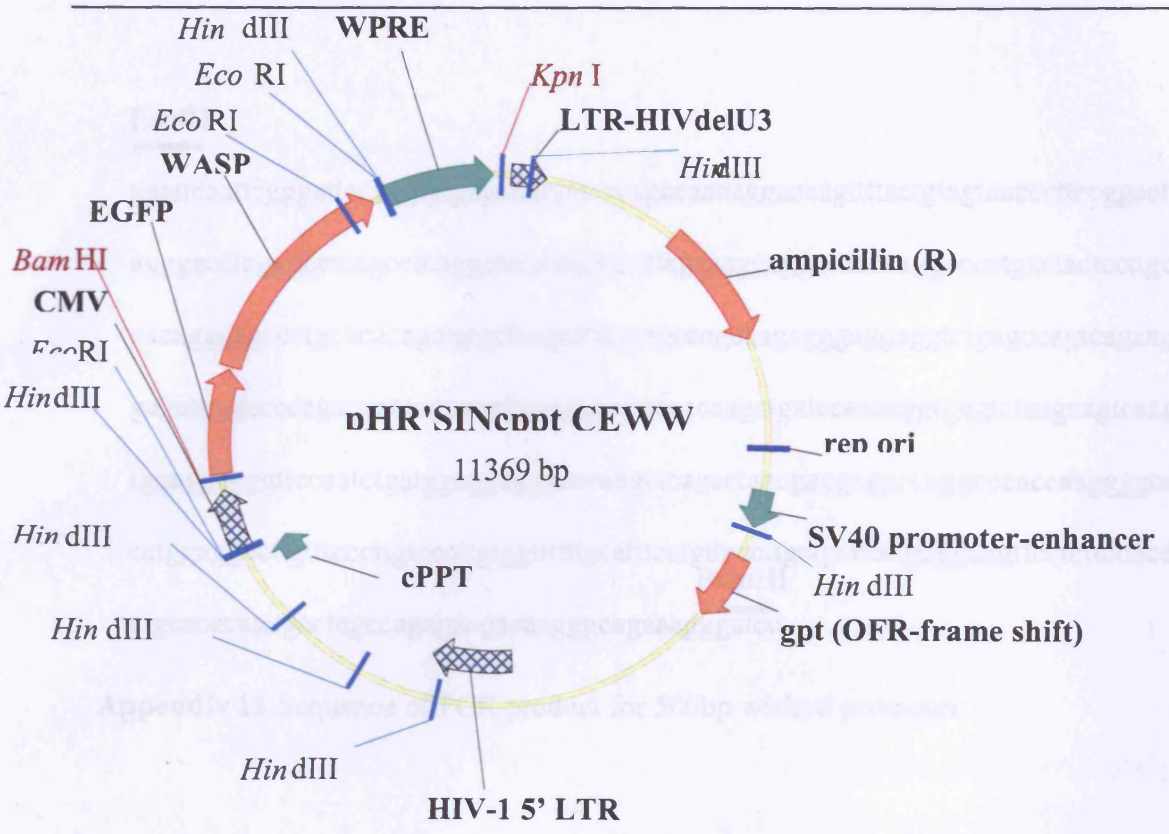
Appendix 6 PINCO-EGFP-WASp retroviral construct



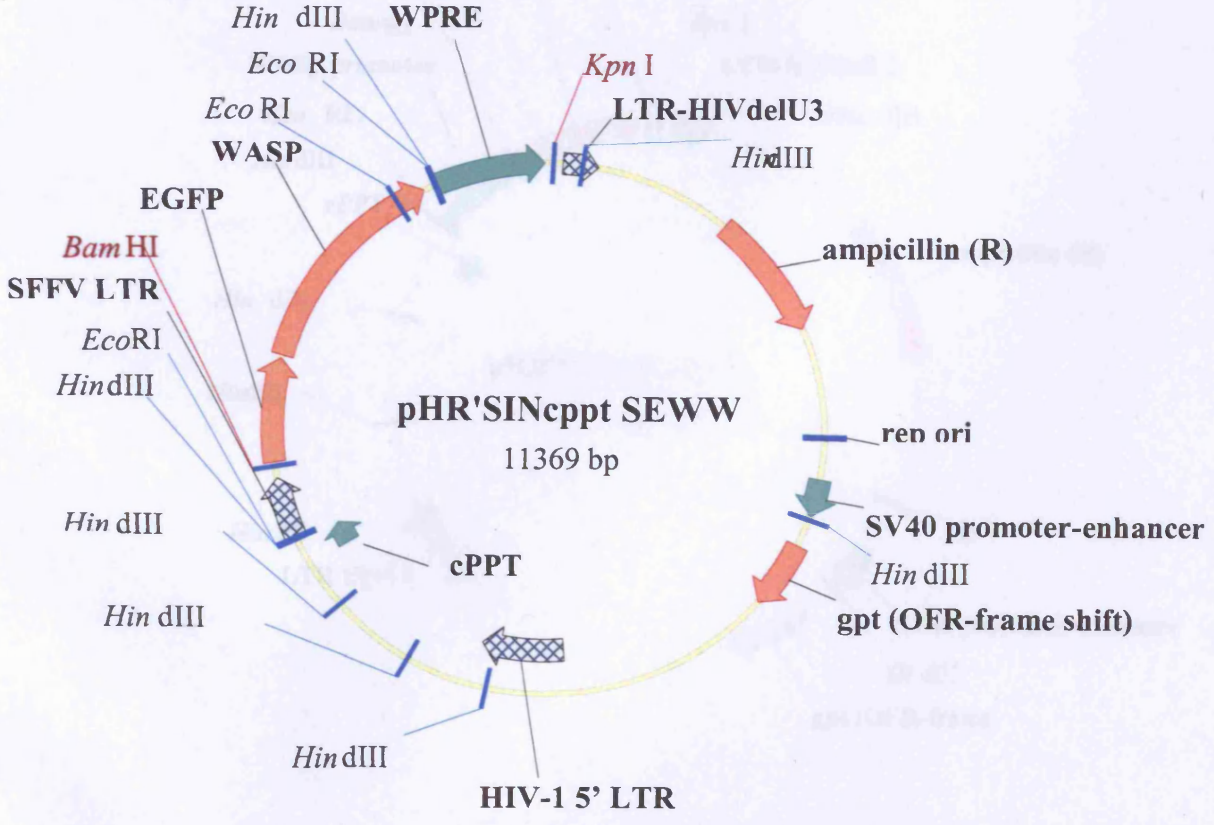
Appendix 7 CMV EGFP WPRE Lentiviral construct



Appendix 8 SFFV EGFP WPRE lentiviral construct



Appendix 9 CMV EGFP-WASp WPRE lentiviral construct



Appendix 10 SFFV EGFP-WASp WPRE lentiviral construct

EcoRI

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BamHI

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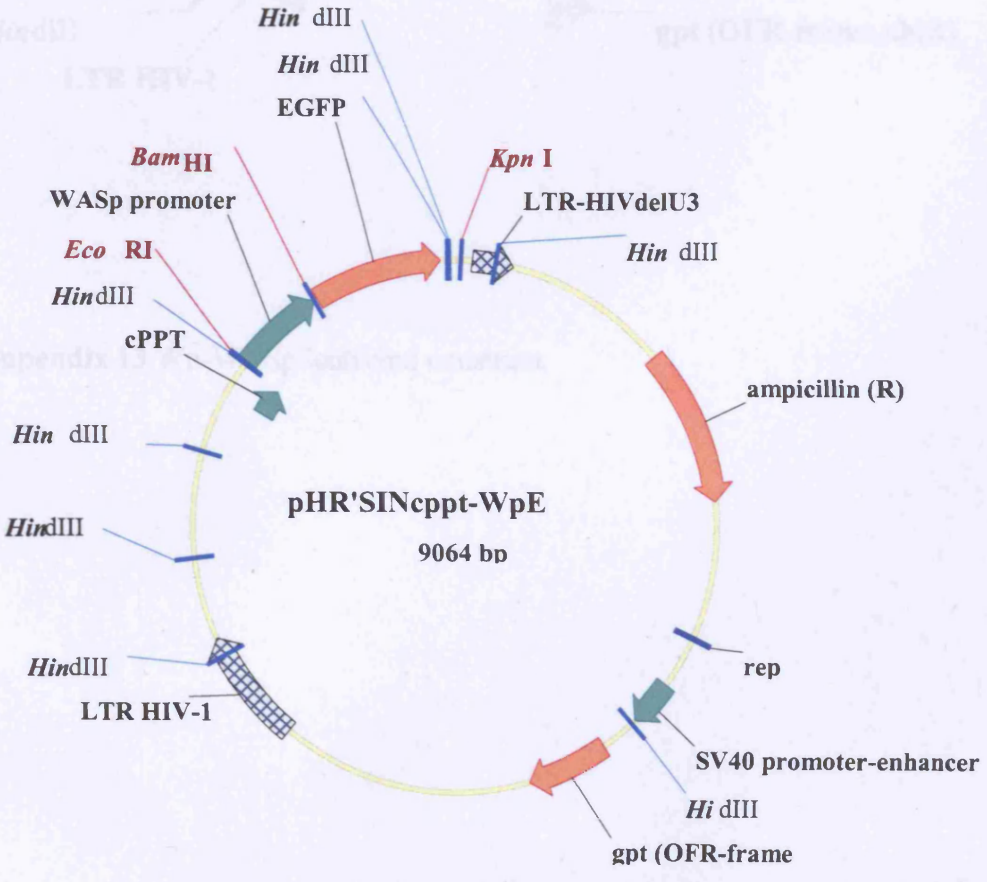
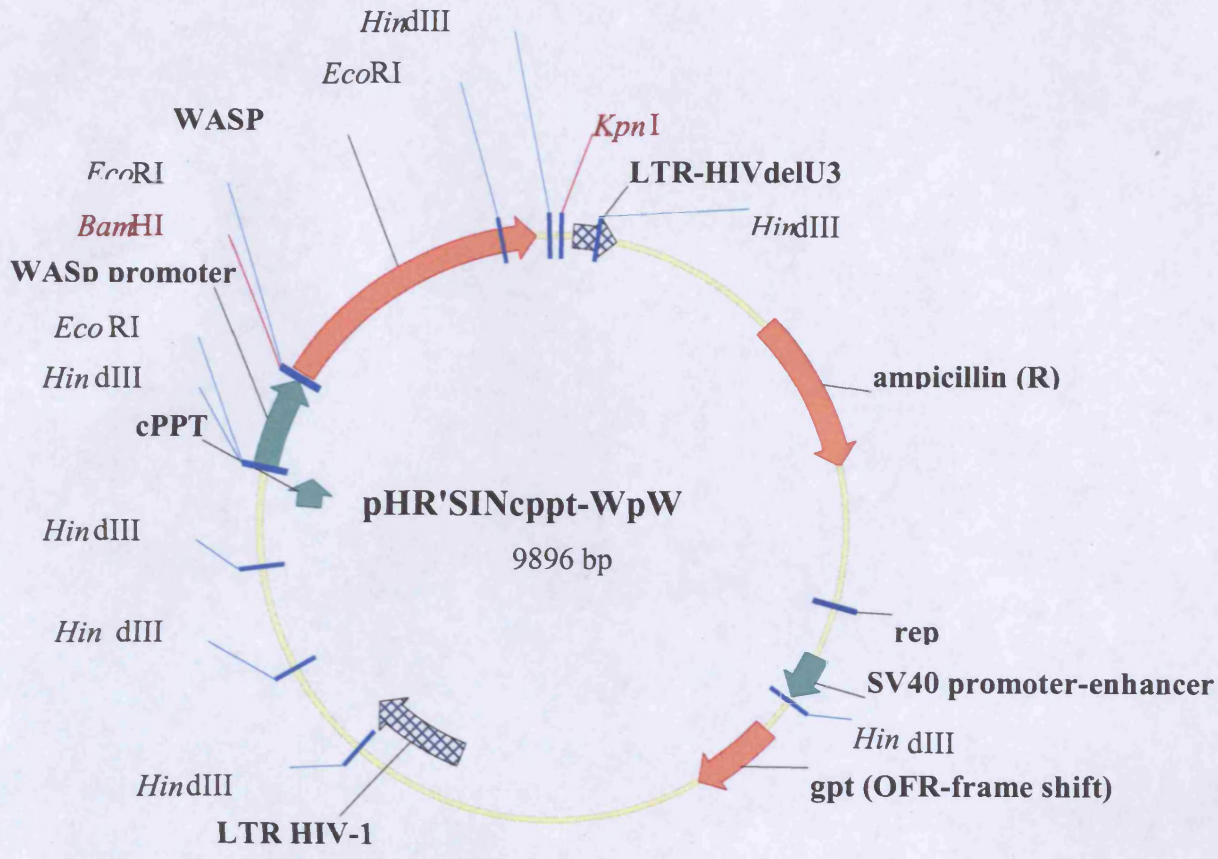
Appendix 11 Sequence of PCR product for 500bp wiskott promoter.



Appendix 12 Wp-EGFP lentiviral construct



Appendix 13 Wp-WASp lentiviral construct